A study of amino acid metabolism in grape berries (*Vitis vinifera* L. Sauvignon blanc)

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by

Scott Michael Gregan

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Amino acids are important primary metabolites and one of the major sources of nitrogen in grapes. Amino acids also occupy a central junction in grapevine biochemistry and are important for vine metabolism and berry homeostasis. The majority of a grapevines photosynthesis and nitrogen assimilation occurs in the leaves, the products of which (sugars and amino acids) are exported to the grape bunches (fruit). In the grape berries themselves, amino acids are the precursors to secondary compounds supporting grapevine growth and physiology, while additionally having an important role in wine quality outcomes. Yet, despite the importance of amino acids in grapevine, their regulation and accumulation in grapes is poorly understood and usually inferred through research in other plant species. Leaf and shoot removal are common practice in commercial vineyards, with any such canopy management interventions having the potential to influence berry biochemical composition. This well-established viticultural intervention was used as an experimental technique to investigate the effect on amino acid biochemistry in Sauvignon blanc grapes.

Basal leaf removal had a significant effect on amino acid accumulation in Sauvignon blanc grape berries. This was reflected in reductions of total amino acid concentrations in the berry, differential accumulation at the level of amino acid families and within families, individual amino acids. Individual amino acids also had differential responses to leaf removal. Aside from the quantitative effect of basal leaf removal on amino acid concentrations, leaf removal also had a qualitative effect on some individual amino acids, modifying their proportions of accumulation in the grape berries. The α-ketoglutarate family of amino acids (glutamine, glutamate, arginine and proline) was further studied, being the family that contains the predominant concentrations of amino acids through berry development and having both quantitative and qualitative aspects in their response to basal leaf removal. Glutamine was the dominant amino acid early in development and decreased throughout. Proline and arginine both increased steadily through development to become most abundant amino acids in grape berries at harvest. Glutamate levels stay relatively consistent through development despite being the direct

precursor of the two most abundant amino acids at harvest (arginine and proline) and contributing as a substrate / co-factor for other amino acids via other metabolic reactions (pathways).

The significant reduction in amino acid concentrations in grape berries due to leaf removal, was hypothesised to be predominantly due to the effect of an altered source/sink balance and partitioning impacts. The accumulation of sugars in the berry was less impacted by the same leaf removal treatments. A model was proposed, whereby the younger leaves further up the shoot can compensate with an increased carbohydrate export to the bunches, but this same compensation is not achieved with respect to amino acids.

The mechanisms of amino acid accumulation in the grape berries was further examined, by investigating a number of genes involved in different aspects of α-ketoglutarate amino acid biochemistry. Investigating transcriptional changes of genes involved in these amino acid metabolic pathways, demonstrated differential expression of a range of transcripts involved in metabolism and regulation of glutamine, glutamate, arginine and proline. These experiments also present novel information, regarding the up-regulation of expression of genes involved in arginine and proline metabolism through berry development. In other plant species, proline is induced as a stress response, but in grapevine, the proline metabolic pathways are not well characterised and the accumulation of proline is poorly understood. An increase in expression of proline biosynthetic genes through veraison was demonstrated with a maintenance of transcript counts at postveraison time points, overlapping the period in berry development when proline accumulation in the berry is increasing and the berry is starting to accumulate larger amounts of sugars. An increase in expression of genes related to proline and arginine degradation pathways was also demonstrated through berry development. This implied that even as the berry is accumulating arginine and proline, there appeared to be the potential for interconversion and turnover of these amino acids through the upregulation of enzymes involved in their catabolism.

The relative activity of an associated enzyme (ornithine aminotransferase) which was transcriptionally up-regulated, was also studied. Enzyme assays performed on treatment samples indicated that ornithine aminotransferase specific activity increased through Sauvignon blanc berry development and ripening. Additionally, the maintenance of a leaf canopy was important in regulating ornithine aminotransferase enzyme activity, as relative specific activity was increased by basal leaf removal compared to control (maintained leaf canopy) samples. This indicated the potential for allosteric regulation of key amino acid pathway enzymes in grape berries.

Overall, the results presented in this thesis substantially improve the understanding of the mechanisms influencing amino acid biochemistry in *Vitis vinifera* L. var. Sauvignon blanc grapes.

Keywords: grapevine, *Vitis vinifera*, Sauvignon blanc, leaf removal, amino acids, glutamine, glutamate, proline, arginine, ornithine, gene expression, enzyme activity, light exposure, assimilate partitioning

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Publications and Conferences

Publications

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Oral presentations

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Gregan SM, Winefield C & Jordan B (2016) Investigating amino acid metabolism and accumulation in Sauvignon blanc grapes in response to canopy manipulation. *IX International Cool Climate Wine Symposium*, Brighton, England.

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Other conferences and research activities

Invited by Plant & Food Research to showcase and present SSIF funded research activities at the Romeo Bragato National Conference 2018.

Gregan SM, Winefield C & Jordan B (2016) Amino acid metabolism and accumulation in Sauvignon blanc grapes – investigating berry composition in response to canopy manipulation. *X International Symposium on Grapevine Physiology and Biotechnology*, Verona, Italy.

Jordan B, Gregan SM, Liu L and Winefield C (2016) The effects of leaf removal and UV-B on grape biochemical composition. *X International Symposium on Grapevine Physiology and Biotechnology*, Verona, Italy.

Student presentation award – Romeo Bragato National Conference 2015 (see above).

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List of Abbreviations

Only abbreviations that appear more than once and within the body of the text are shown.

Abbreviations within figures or tables are explained in the corresponding legends.

Vv	*Vitis vinifera*	ABA	abscisic acid
OAT	ornithine aminotransferase	GSA	glutamate-semialdehyde
DNA	deoxyribonucleic acid	P5C	pyrroline-5-carboxylate
GS	glutamine synthetase	*P5CS*	P5C synthetase
GOGAT	glutamate synthase	*P5CR*	P5C reductase
YAN	yeast assimible nitrogen	*PDH*	proline dehydrogenase
cDNA	complementary DNA	*P5CDH*	P5C dehydrogenase
NADH	nicotinamide adenine dinucleotide	NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide	*NAGS*	*N*-acetylglutamate synthase
Fd	ferredoxin	UV	ultraviolet
TSS	total soluble solids	RNA	ribonucleic acid
DPV	days postveraison	PCR	polymerase chain reaction
DOY	day of the year	*FLS4*	flavonol synthase 4
GDD	growing degree days	RIN	RNA integrity number
LTA	long term average	rcf	relative centrifugal force
SNAC2	stress-responsive NAC2	lsd	least significant difference

Chapter 1
Introduction

1.1 Research background and rationale

The domestic grape growing and wine industry in New Zealand has grown rapidly over the last two decades and in 2018 was the largest horticultural export by value. New Zealand wine is exported to over 80 countries with an export value of NZ$1.7 billion, an increase of 40% since 2013 alone (NZ Winegrowers 2018).

Vitis (V) vinifera var. Sauvignon blanc is the major commercial grape variety grown in New Zealand and the most important cultivar for the New Zealand wine industry. Of the 37969 hectares of grapevine planted in New Zealand, Sauvignon blanc accounts for 23102 hectares. This represents 71% of the entire New Zealand grape production in 2018. The next closest variety in terms of tonnes produced is Pinot Noir, which represents 8% of total grape production. The Marlborough region of New Zealand specifically, produces high quality Sauvignon blanc that is internationally recognized for its unique flavour and aroma characteristics. Because of the success of Marlborough Sauvignon blanc, its plantings have continued increasing year-on-year to currently contribute 81% of the total Sauvignon blanc plantings in New Zealand (NZ Winegrowers 2018). 86% of New Zealand's wine exports are Sauvignon blanc, emphasizing the importance to the industry of this single variety.

Because of the importance of Sauvignon blanc to the New Zealand wine industry, grape growers, viticulturists, wine makers and researchers have been working together to improve their understanding of the mechanisms controlling grape composition. A multicentre, multidisciplinary research programme was initiated in 2004 (and then renewed in 2010), researching the science of Sauvignon blanc. The programmes were a collaboration between New Zealand Winegrowers (plus industry partners) and three major research providers: Lincoln University; the University of Auckland; and Plant & Food Research. One of the key areas of research undertaken at Lincoln University has been to understand nitrogen assimilation and amino acid metabolism along with the relevance to grape production and subsequent wine-making.

The French word, veraison, has been adopted to describe the onset of ripening and is the major developmental switch in grapes. Berries change from being small, acidic and hard, to a status where they start to accumulate water and sugars, become larger, softer, less acidic and coloured (in the case of red grapes). The most dramatic changes in the berries biochemical composition, occurs postveraison in the ripening phase. Even though the berry approximately doubles in size between veraison and

harvest, when many solutes are diluted out due to an increase in berry volume, total amino acids in particular, continue to accumulate substantially.

Amino acids are important primary metabolites and are one of the major sources of nitrogen in grapes and they are particularly important in the wine-making process as primary sources of nitrogen during yeast fermentation. Amino acids therefore are at a central junction in grape biochemistry homeostasis and potential wine quality dynamics. Amino acids are also precursors of secondary compounds that are linked to flavour and aroma of wine, whether that be directly (such as flavonols and methoxypyrazines) or existing as precursors with then the subsequent aid of yeast fermentation. Although grape berries can assimilate nitrogen, the majority of nitrogen assimilation is generally considered to occur in the leaves, subsequently exported into berries in an amino acid form. Therefore, given that leaf and shoot removal and other canopy manipulations are common practice in commercial vineyards, any such canopy management or other viticultural interventions have the potential to influence grape berry biochemical composition and ultimately, wine composition and quality.

Despite the importance of amino acids in grapes, their regulation and accumulation in the grape berry is poorly understood and usually inferred through research in other plant species. For example, previous research shows that mature grape berries accumulate high concentrations of the amino acids arginine and proline, and these together make up the highest proportion of the total amino acid concentration in the grape at harvest. The regulation of free proline and arginine accumulation in ripening grapes has not been extensively studied, as well as the function(s) that these amino acids have during grape development. In addition, from a grapevine research perspective, insufficient attention has been paid to defining the genes that regulate proline and arginine accumulation/degradation during grape development.

The aim of the research presented in this thesis is to determine the effect of preveraison and postveraison leaf removal on the accumulation of amino acids in Sauvignon blanc grapes during berry development. Using grape samples from leaf removal experiments, genetic regulation of nitrogen assimilation and amino acid metabolism in developing Sauvignon blanc grapes was also investigated, with a particular focus on glutamine, glutamate, proline and arginine metabolic pathways (Figure 1.1). Also investigated, was the mechanism of proline accumulation as a result of a potentially active alternative pathway through arginine and ornithine intermediates. The specific objectives and gaps in the literature being addressed by this thesis are described in detail in Chapter 1.11.

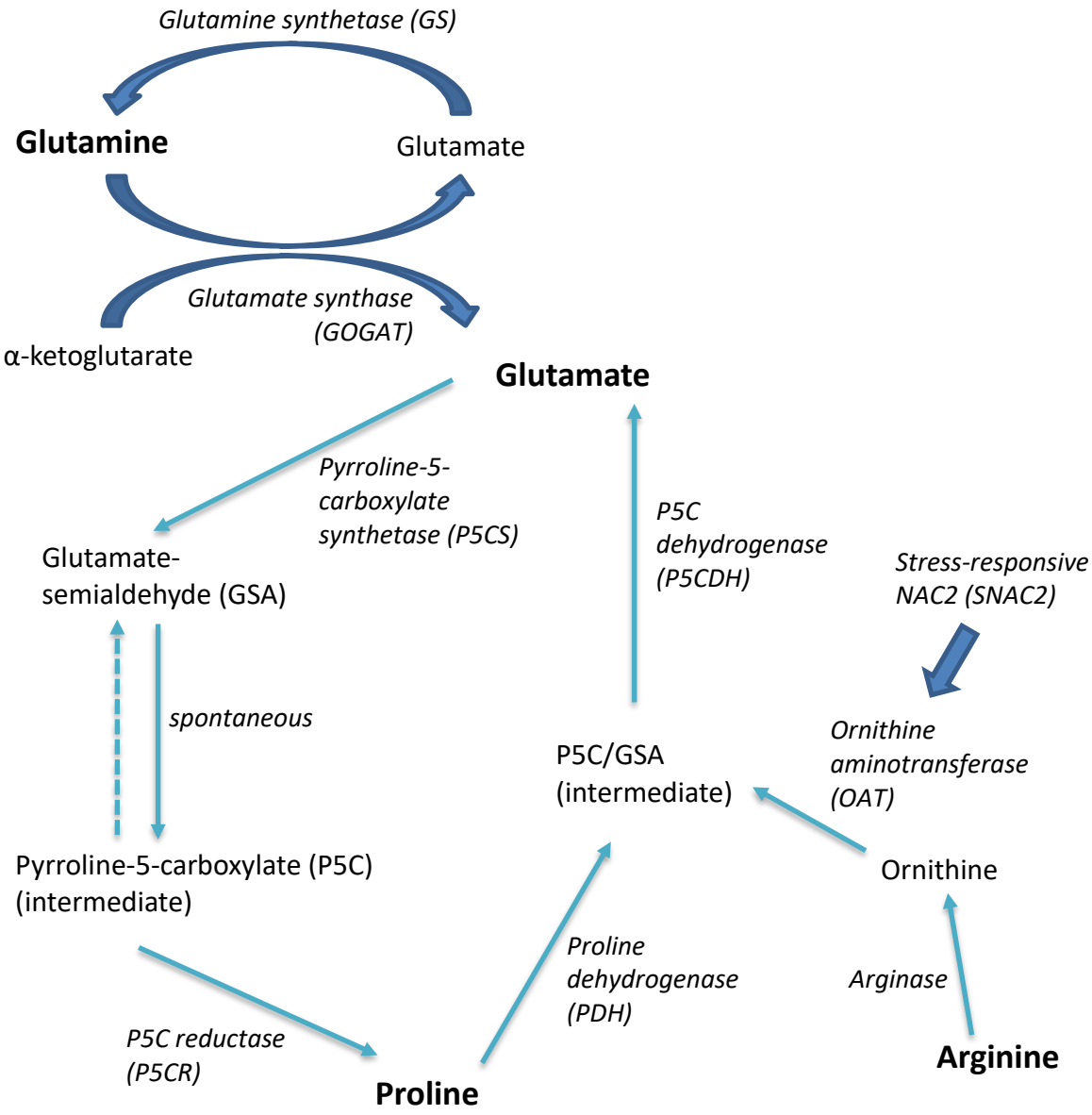


Figure 1.1 The metabolic pathways of the α-ketoglutarate family of amino acids.

Simplified schematic showing the metabolic pathways of glutamine, glutamate, arginine and proline, the regulatory molecules involved (*in italics*) and some linkages between the pathways.

1.2 The commercial importance of grapes

The grapevine (*Vitis* species) has a significant place in human history and is now cultivated worldwide and established as one of the world's most important fruit crops. Grapevines (fruit and leaves) are used in a wide variety of food products from fresh fruit and leaves, dried fruit (raisins and sultanas), juice and wine.

Evidence for the early domestication of grapevines and pottery wine residues indicate that grape growing for food and wine production was well distributed as early as the Neolithic periods (approximately 10000 BC) in the areas around the Mediterranean, Black and Caspian Sea's (McGovern et al. 2004). Considerably later, the Romans and Greeks freely spread (*V. vinifera*) grape cultivation and wine production throughout Europe and North Africa and subsequently, European colonial expansions introduced *V. vinifera* to the Americas, Asia, Australia and New Zealand.

The worldwide area planted under grapevines that are destined for the production of wine or table grapes and raisins was 7.5 million hectares in 2016 (OIV 2017). This is marginally reduced from 7.8 million hectares at the turn of this century in 2000. Nevertheless, even with slightly less plantings, global grape production has increased from 64.8 million metric tons in 2000 to 75.8 million metric tons in 2016. The majority of grapes are made into wine and related fermented products. In 2015, approximately 47% of world production was used to produce wine, 36% for fresh grapes, 8% for dried grapes and 6% for grape juice (OIV 2017). The economic importance of wine is enhanced by the fact that historically, wine has become an integral accompaniment to daily cuisine and quality wine has become a highly valued commodity comprising an enormous array of styles and price points. Today, wine and grapes are the most economically important fruit products globally and it is estimated that there are more than 5000 distinct cultivars of *V. vinifera* grown worldwide (Keller 2015).

1.3 Classification of grapevines and *Vitis* species

Grapevine are a diverse species which belong to the botanical family Vitaceae, which broadly includes other woody shrubs that possess leaf-opposed tendrils to grow and climb vertically. Within the Vitaceae family, the *Vitis* species define the "modern grapevine" and consist of populations of vines that have distinguishable morphological traits and evolutionary isolation through geographical and ecological barriers (Jackson 2014; Keller 2015). A description of the botanical classification of grapevine is shown in Table 1.1.

Table 1.1 The botanical classification of grapevines.

Classification	Overview
Domain Eukarya	
Kingdom Plantae	Have a haplo-diploid life cycle and cell walls made from cellulose.
Phylum Angiospermae	Flowering plants. Have the most complex reproductive system of the plants. The fruit grows from the ovary embedded inside a fertilised flower.
Class Dicotyledoneae	Dicot plants start their life cycle with two cotyledons (first leaves) in the seed.
Order Vitales	A small order of flowering plants that contains a single family: Vitaceae.
Family Vitaceae	Members of Vitaceae are collectively called grapevine and are typically climbing shrubs and woody lianas.
Genus *Vitis*	Members are perennial vines or shrubs with tendril-bearing shoots.

Summarised from Keller (2015).

The Genus *Vitis* is composed of approximately 70 species and modern grape growing and winemaking utilise the different species for distinct commercial purposes. Nevertheless, it is the Eurasian *V. vinifera* species that are the most cultivated grape varieties worldwide and also the most important for modern wine production. Other *Vitis* species such as the American *V. rupestris* or *V. riparia* are not typically used for fruit production, instead these species are often utilised for their other traits such as a natural resistance to grapevine diseases such as phylloxera (Terral et al. 2010). This makes them commercially important for breeding and their use as rootstocks, on which the *V. vinifera* wine varieties are grafted onto.

1.4 Annual growth cycle of the grapevine

Grapevine is a perennial plant with its growth cycle and fruit production extending over two concurrent growing seasons. The growth of a shoot begins as development of a (latent) bud in the first year. After a period of winter dormancy, budbreak occurs and a shoot emerges. Seasonal growth of the shoot is driven by increasing day length and air temperature and energy is supplied from stored carbohydrate, as no photosynthesis is yet occurring in the newly emerging leaves (May 2000; Winkler et al. 1974). The modified Eichhorn and Lorenz (E-L) system has been widely used as a reference to identify distinct developmental stages in grapevine growth and is shown in Figure 1.2.

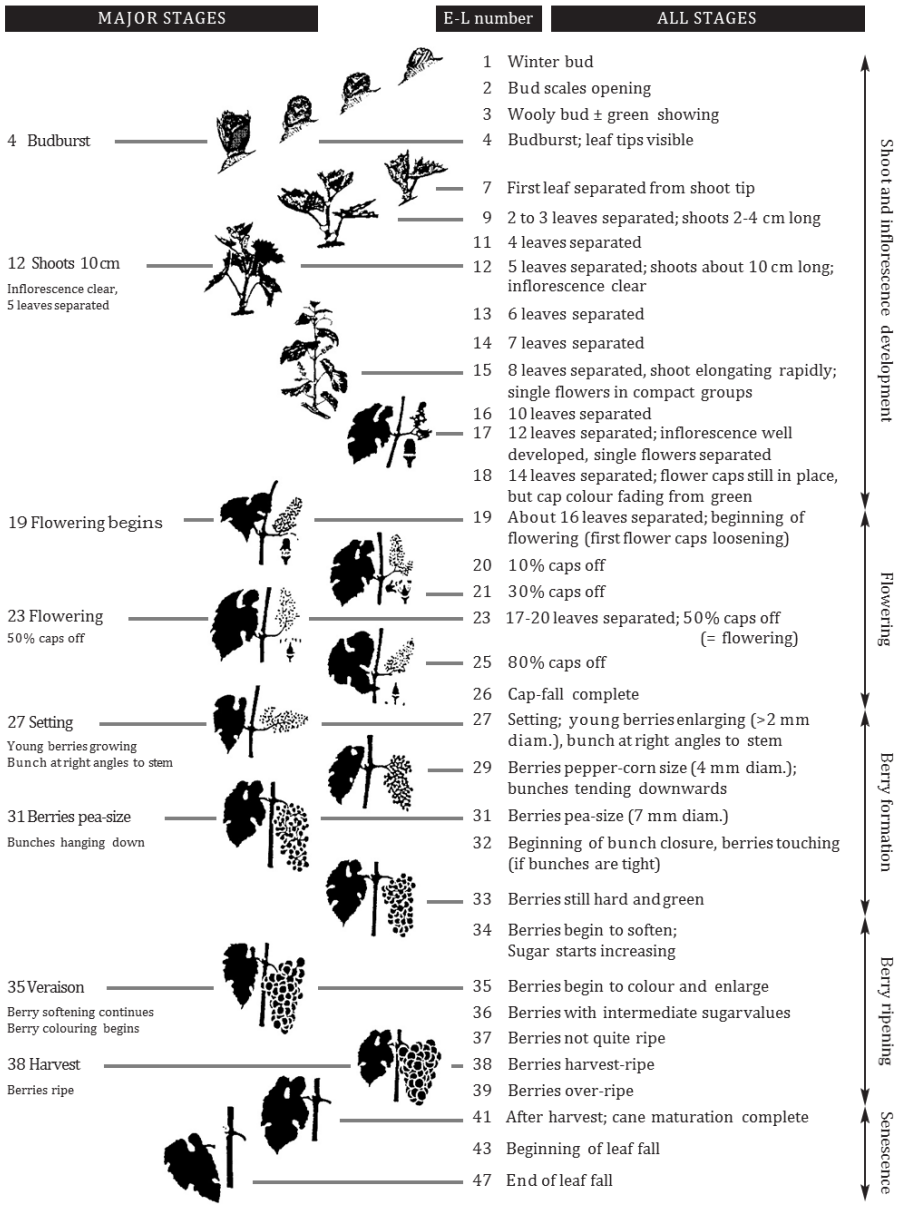


Figure 1.2 Annual growth cycle of the grapevine.

Key stages of grapevine growth and development according to the modified Eichhorn and Lorenz (E-L) system (from Coombe (1995)).

1.4.1 Fruit development and berry growth

Once developing leaves are capable of supplying carbohydrate needs to the vine, flowering and fertilisation begins. Berry expansion is rapid following fertilisation and first occurs through cell division and then later by cell expansion. Grape berry growth consists of two successive sigmoidal growth periods separated by a lag phase (Coombe & McCarthy 2000). During the first growth period, following rapid cell division, the berry expands in volume as solutes accumulate (Possner & Kliewer 1985). The second phase of berry growth is at veraison and is characterized by softening and pigmentation appearing in the skin (in red cultivars) of the berry (Figure 1.3). Increases in berry volume (primarily due to water uptake) during this second phase are associated with carbohydrate accumulation postveraison. Many other solutes that accumulated in the grape berry during the first period of development remain postveraison, but due to increases in berry volume, their concentrations are significantly diluted.

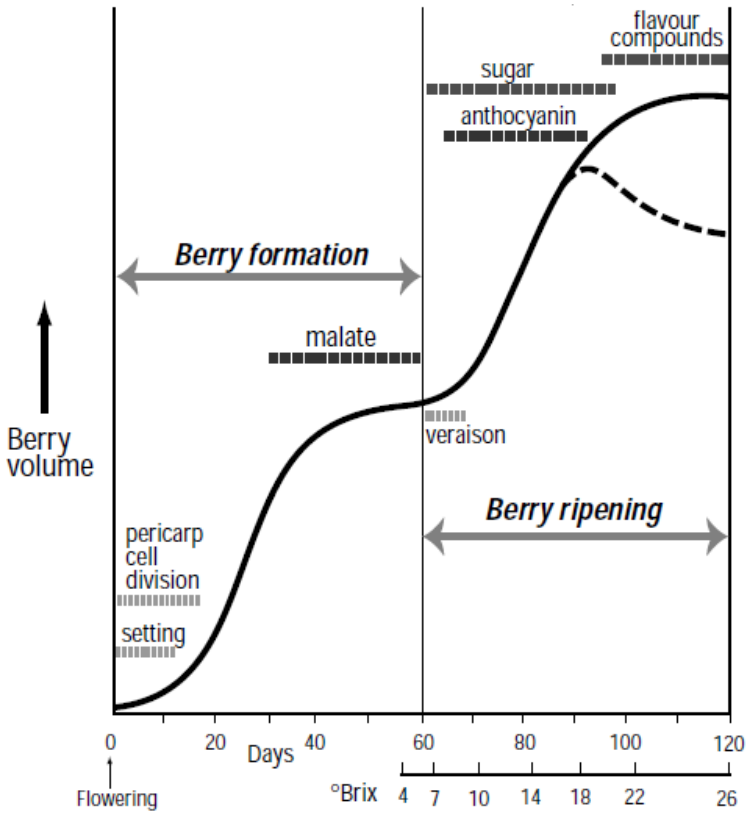


Figure 1.3 The major developmental stages of a maturing grape.

A schematic of berry growth and development illustrating periods of biochemical accumulation and TSS accumulation (°Brix) (from Coombe & McCarthy (2000)).

The grape berry contains three main types of tissue; skins, seeds and pulp (Figure 1.4). The different tissues vary considerably in composition throughout development. The cells of the skin tend to accumulate phenolic compounds such as flavonols and anthocyanins in relatively high amounts as the grape matures. The pulp is the primary site for the accumulation of sugars, primary acids (tartaric and malic) and amino acids. The seeds generally have considerably less of the aforementioned compounds, but do contain relatively high concentrations of phenolic hydroxycinnamates and tannins (Jackson 2014).

The distribution between tissue types of important biochemical components in the grape berry, such as amino acids and phenolic compounds, can differ greatly between variety, environmental and seasonal effects. Additionally, there can be a great deal of variability between berries within a cluster, between clusters within a vine, between vines within a cultivar and between vines within a vineyard (Boss & Davies 2001; Kennedy et al. 2000; Stines et al. 2000).

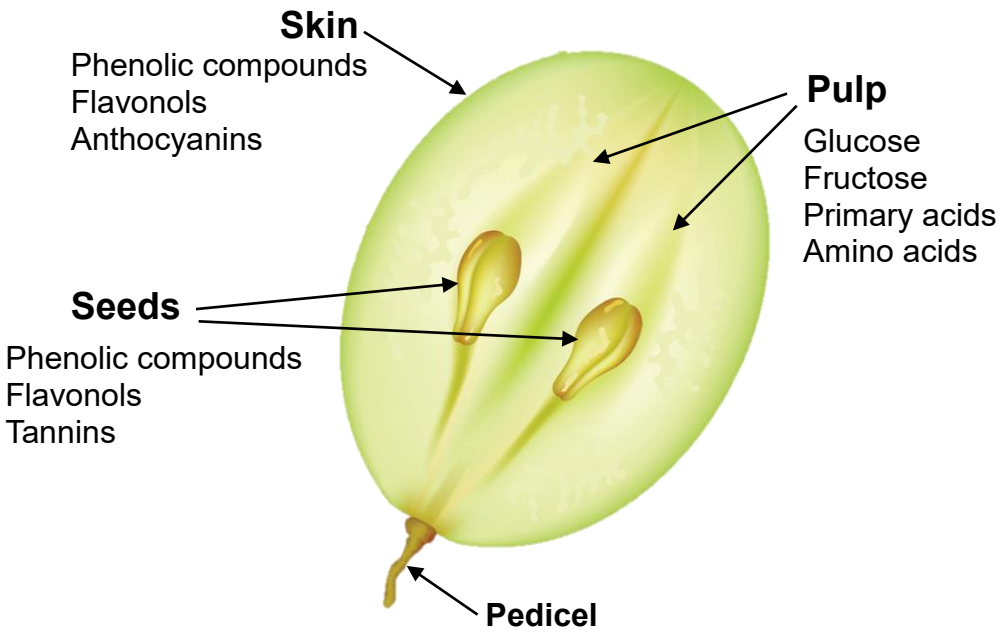


Figure 1.4 Cross-section of a mature grape berry.

Schematic structure of a mature grape berry showing distribution of biochemical composition between the major tissue types; skin, seeds and pulp.

1.5 The grapevine, nitrogen and amino acids

1.5.1 The importance of nitrogen

Of all of the mineral nutrients, nitrogen often is the most important in terms of influencing grapevine growth and physiology. Crucially, nitrogen is a chemical component of most of the critically important plant biochemical compounds, including nucleic acids which hold the plants genetic information contained in a grapevines DNA (deoxyribonucleic acid). Amino acids contain large amounts of a plants nitrogen, both in a free form and linked together in the form of polypeptides, proteins and enzymes, the latter of which drive all of the biochemical reactions of cells metabolism.

But how much nitrogen is good for the vine and beneficial to grape berry composition and wine quality? Manipulation of grapevine nitrogen (and other nutrition) has the potential to significantly influence berry biochemistry and composition and ultimately the composition and quality of resulting wine. The answer is - it depends on a wide range of factors including cultivar/rootstock, climate, trellis system, canopy shading and timing and amount of nitrogenous fertiliser supplementation (Bell & Henschke 2005; Bell & Robson 1999; Huang & Ough 1989; Kliewer & Cook 1971; Miele et al. 2000; Smart et al. 1988; Spayd et al. 1994; Stines et al. 2000)

1.5.2 The grapevine nitrogen status

The vegetative and grape yield responses of the grapevine depend on the initial nitrogen status of the vine prior to supplementation, and subsequent application of nitrogen will set off a series of reactions that may directly or indirectly impact grape berry biochemistry and result in influencing wine quality.

When a vines nitrogen status is deficient, supplementation with nitrogen stimulates nitrogen metabolism, protein biosynthesis and related biochemistry. This generally has a positive effect on vine growth, vigour and berry composition (Bell & Robson 1999; Kliewer & Cook 1971). A grapevines nitrogen status is considered to be sufficient when maximal growth, yield and berry composition is attained. Further additions of nitrogen in this case do not increase growth and more importantly yield past this optimal level. In fact, overuse of nitrogen supplementation may have detrimental effects on vine growth and grape yield/composition. High nitrogen status can disrupt vine balance, source-sink relationships and canopy microclimate through excessive vegetative growth (Bell & Henschke 2005; Kliewer & Cook 1971). Increasing vegetative vigour increases canopy density, which results in a change in the bunch microclimate and alters a variety of environmental factors including light exposure/shading, ultraviolet (UV) radiation, bunch temperature and humidity (Smart 1985). The source-sink balance, light and temperature in particular influence berry biochemistry and grape quality (Iland 1989).

1.5.3 Amino acids and grape berry development

The impact of nitrogen application on berry biochemistry and grape composition/quality, is the combination of its direct effect on vine growth and metabolism and subsequent indirect effects due to the influence of vegetative vigour and yield. The main effect on grape quality components is to increase total nitrogen concentration and nitrogenous compounds, namely arginine, proline, other free amino acids and ammonium (reviewed in Bell & Henschke (2005) and references within).

In the grape berry, nitrogen is found in mineral (inorganic) forms ($NH4^+$, $NO3^-$, $NO2^-$) and organic forms (primarily, amino acids and proteins). This nitrogen is also called fermentable nitrogen under normal winemaking conditions, which yeasts consume during alcoholic fermentation. Inorganic nitrogen in the form of ammonium can represent up to 80% total nitrogen in the grape berry before veraison, but declines significantly during ripening (Stines et al. 2000; van Heeswijck et al. 2001). Amino acids on the other hand, increase through veraison and ripening to harvest. Of the total nitrogen at harvest, 50-90% is in the form of free amino acids (Hernandez-Orte et al. 1999). Early in the development of the grape berry, glutamine is easily the dominant amino acid in growing berries, presumably transported into the berry as the major transport molecule for nitrogen (Gregan et al. 2012; Keller 2015; Stines et al. 2000). Once in the berry, glutamine is a precursor for a variety of biosynthetic pathways and is converted to other amino acids via the action of the glutamine synthetase - glutamate synthase (GS-GOGAT) pathway (see Chapter 1.6.3).

As the grape berries ripen and mature, the two amino acids of arginine and proline in particular, accumulate to high concentrations and make up the highest proportion of total amino acids by harvest (Gregan et al. 2012; Stines et al. 2000). Arginine is important to berry homeostasis as a store of nitrogen, but also as a preferential source of nitrogen for yeast fermentation (Roubelakis-Angelakis & Kliewer 1992). The substantial accumulation of proline in grape berries that takes place postveraison and in the later stages of development is poorly understood, but is directly important to the wine-making process. Conversely to arginine, the high level of nitrogen incorporated into proline cannot be utilised by most commercial yeast strains during a normal anaerobic fermentation (Duteurtre et al. 1971).

Amino acids therefore occupy a central junction in grapevine biochemistry and are important for vine metabolism and berry homeostasis. The concentration of each amino acid in the grape berry can vary significantly depending on the grape cultivar, rootstock, season, viticultural practices and developmental stage (Bell & Henschke (2005) and references within). Consequently, the amino acid content of grapes and musts can be hugely varied at harvest time.

1.5.4 Amino acids and wine quality

The primary determinant of juice/must composition before fermentation to wine, is the grape berry composition at harvest. The most dramatic changes in the berries biochemical composition, occurs postveraison during the ripening phase. Even though the berry approximately doubles in size between veraison and harvest, when many solutes are diluted out due to an increase in berry volume, total amino acids continue to accumulate substantially. Therefore, it is not surprising that amino acid accumulation in the grapes/must and its subsequent utilisation by yeasts during fermentation, will have significant influences on wine quality. Indeed, this has been shown to be the case.

At harvest, the amino acid profiles of grapes are dominated by arginine and proline, with lesser concentrations of other amino acids in various proportions (Hernandez-Orte et al. 1999; Hilbert et al. 2003; Rodriguez-Lovelle & Gaudillere 2002). Amino acids differ in their efficiency as nitrogen sources for yeast fermentation and individual amino acids are precursors of aroma and flavour compounds in wine (Bell & Henschke 2005; Huang & Ough 1989; Jiranek et al. 1995). Thus, the regulation of amino acids is not only important for berry homeostasis, but also, their harvest profiles are key components in determining wine quality (Figure 1.5).

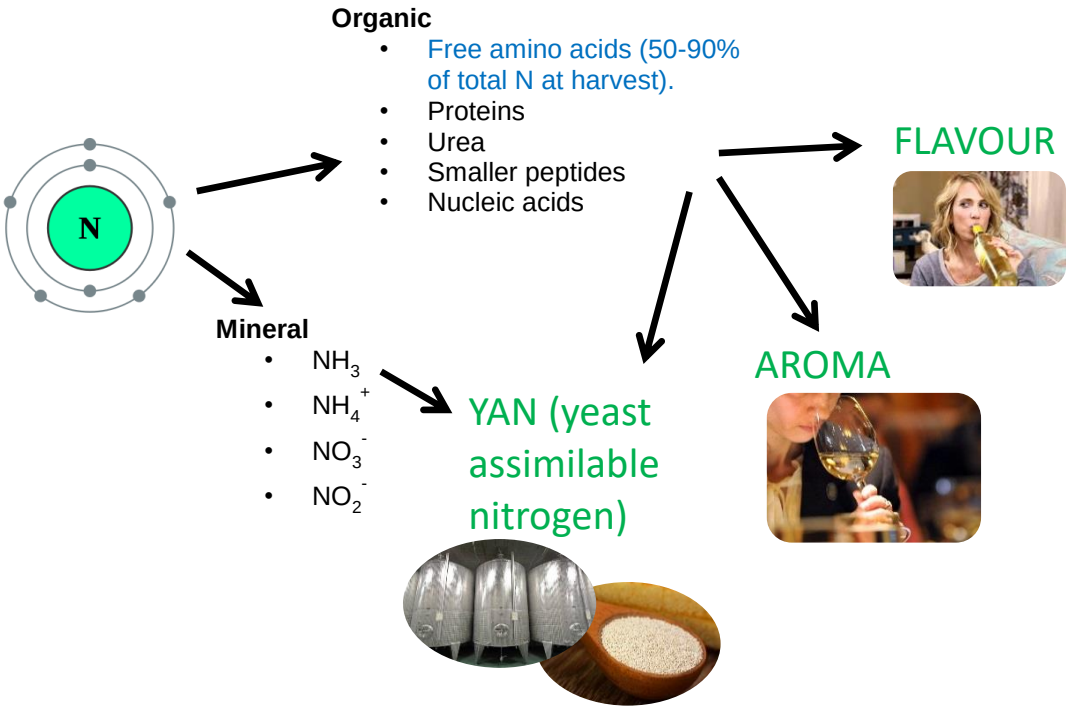


Figure 1.5 The principal sources of nitrogen in the grape berry.

The various sources of nitrogen in a mature grape berry, differentially contribute to yeast fermentation and wine quality.

1.5.5 Amino acids, fermentation kinetics and yeast assimilable nitrogen

The principal yeasts used for the fermentation of grape juices/musts into wine (normally *Saccharomyces cerevisiae* species), utilise inorganic nitrogen sources of ammonium and organic (nitrogen) sources of primary amino acids for their growth. Nitrogen in the form of secondary amino acids such as proline for example, cannot be metabolised under usual winemaking conditions (Duteurtre et al. 1971).

Primary amino acids therefore comprise an important usable fraction of the yeast assimilable nitrogen (YAN) content of grape juice. If amino acid concentrations are not optimised then the result can be a "stuck ferment" (in the case of low YAN concentrations), where the yeast exhausts available YAN sources and become dormant before the fermentation has completed. Conversely, high YAN concentrations can also lead to increased yeast biomass, fermentation kinetics and formation of undesirable compounds in the wine (Poni et al. 2018). A ratio of the two amino acids proline and arginine, can provide an indication of the proportion of non-YAN (proline) to YAN (arginine), and reflects the potential nutritional value of grape juice (Bell & Henschke 2005). Different cultivars can have significantly different arginine to proline ratios, even though having the same total amino acid concentrations. But even within a single cultivar, this ratio will be influenced by a variety of developmental and environmental factors (Kliewer & Ough 1970; Stines et al. 2000).

1.5.6 Amino acids and flavour aroma differences

Amino acids occupy a biologically pivotal position between primary and secondary metabolism. Aside from the effect on fermentation dynamics, there is also a body of literature investigating amino acid composition in grapes and correlating composition differences to wine quality (Bell & Henschke 2005; Hernandez-Orte et al. 2002; Rapp & Versini 1996). Taste intensity and aroma quality of wines tends to increase as the nitrogen content of the grapes increases (Rapp & Versini 1996). The amino acid profile of a grape has shown to be closely related to the wine quality, in terms of compounds that are derived from amino acid precursors (Hernandez-Orte et al. 2002).

Amino acids are the starting precursors for important secondary compounds in grapes including methoxypyrazines, phenolic compounds, thiols, esters, higher alcohols, flavonols and anthocyanins. Methoxypyrazines and sulphur-containing thiols for example, provide the characteristic aroma of New Zealand (Marlborough) Sauvignon blanc (Lund et al. 2009; Parr et al. 2007). Methoxypyrazines are somewhat distinct in that they exist in grape berries as volatile, free compounds and their final concentration in grapes is highly correlated to methoxypyrazine concentration in wine (Roujou de Boubee et al. 2002). Many other compounds derived from amino acid precursors which play a major role in defining the flavour and aroma profile of a wine (thiols, for example), require biochemical modifications by yeasts during fermentation. Therefore, any differences in amino acid composition of

grape juice, that can influence yeast growth and fermentation dynamics, will impact wine quality and sensory composition. Hernandez-Orte et al. (2002) investigated correlations between amino acid profile and wine aroma in different grape varieties and even venture to suggest that "most vintage and geographical variations observed in wine fermentative aroma are due to the differences in the must amino acid composition induced by the vintage and geographical factors". Their research also identified threonine and to a lesser extent phenylalanine, having the greatest influence on the wine aroma composition. However, a number of other amino acids have been correlated to differences in wine quality components, and are summarised in Figure 1.6.

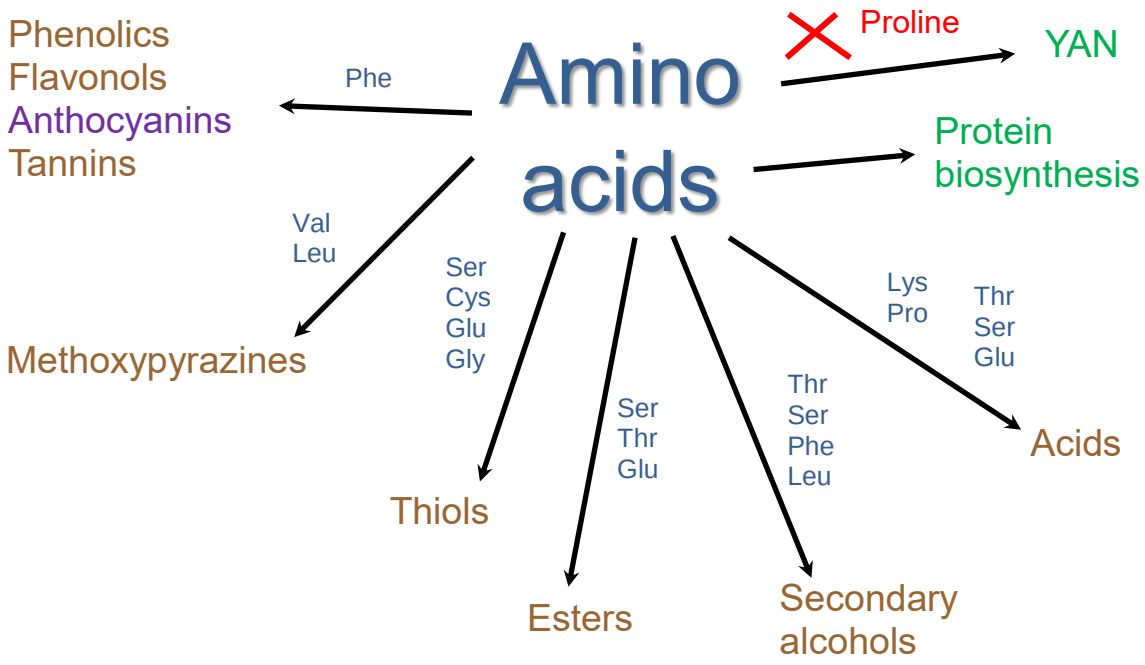


Figure 1.6 Amino acid composition in grapes is correlated to differences in wine quality.

(Summarised from Bell & Henschke (2005); Hernandez-Orte et al. (2002); Rapp & Versini (1996))

The amino acid concentration in wine is generally much less than the initial juice before fermentation, presumably as it is consumed from the metabolic activities of the yeast. At very high concentrations, proline has been shown to impart a sweet taste and arginine, a bitter taste. However, with the exception of proline, most amino acids are at low concentrations in wine and considered to have little direct sensory impact (Hufnagel & Hofmann 2008).

1.6 Nitrogen assimilation

Nitrogen assimilation is the vital biological process in plants in which inorganic nitrogen is incorporated into organic forms, namely amino acids. Glutamine is the main organic nitrogen transport molecule in grapevine, moved through the xylem transpiration stream. With increasing nitrogen supplied to the roots of vines, nitrogen in the form of nitrate is preferentially transported to growing shoots in addition to lesser amounts of glutamine (Keller 2015).

In grapevine, the primary site of nitrogen assimilation is in the leaves. This is predominantly because nitrogen uptake and assimilation in the roots is energy expensive and this energy requirement can be offset by the photosynthetic capability of the leaves (see Chapter 1.6.2). Nevertheless, glutamine, is the principal amino acid transport of nitrogen into grape berries and once in the berry is converted into other amino acids via a network of varied and frequently overlapping biochemical pathways. Whatever the fate of the assimilated nitrogen in these metabolic interconversions, it must still be effectively incorporated into an organic form from the inorganic sources available to plants in the environment. The processes involved in nitrogen assimilation recruits a complex network of biochemical molecules from transporters to enzymes to facilitate the biochemical reactions required.

1.6.1 Nitrogen uptake - reduction of nitrate

Ammonium (NH_4^+) and nitrate (NO_3^-) ions are the most important (and common) forms of inorganic nitrogen in soils available to plant roots for uptake and most plants including grapevine, can utilise either form. In aerobic soils where nitrification (the biological oxidation of ammonium to nitrate and an important step of the nitrogen cycle in soil) is able to occur, nitrate is preferentially taken up by the roots using specific nitrate transporters localised in the roots cell membranes (Miller et al. 2007; Tischner 2000). Absorbed nitrate can be utilised directly in the root cells or alternatively, translocated to shoots and leaves via the xylem. This is dependent to a large extent on supply and demand. With increasing nitrogen concentrations supplied to the root zone, the amount of nitrate that can be reduced by the roots locally is exhausted and excess nitrate is increasingly transported away for assimilation by the shoots and leaves (Sechley et al. 1992).

In plant cells, nitrate reduction is carried out in two steps. In the first step, nitrate is reduced to nitrite (NO_2^-) by the enzyme nitrate reductase, which uses NADH or NADPH as a cofactor depending on the isoform. The second step reduces nitrite to ammonium, catalysed by nitrite reductase. The primary pathway of ammonium incorporation into amino acids is via the glutamine synthetase/glutamate synthase (GS-GOGAT) cycle (Miflin & Lea 1976).

1.6.2 Energy requirement of nitrogen assimilation

Nitrogen uptake and assimilation is intensive in terms of the energy production needed and carbohydrate requirements, metabolism of the latter driving the supply of carbon backbones required for amino acid biosynthesis. When conditions allow, ammonium is assimilated and incorporated into amino acids at the root site itself, thereby avoiding the transport of organic compounds (carbon backbones) down to the roots from the (more active photosynthetic) leaves, to "collect" the nitrogen and return it back as amino acids. If the supply of nitrate increases, the roots assimilation activity is unable to keep up with supply, and so transports excess nitrate to the leaves for assimilation (Keller et al. 1998; Vidmar et al. 2000).

This can constitute a problem for the grapevines ability to ripen developing fruit in terms of changing the canopy, fruit microclimate and altering local metabolism in the shoots and leaves. Increasing nitrogen supply to the leaves will enhance vegetative growth and hence, photosynthesis. While this can have an initial positive effect on carbohydrate availability for ripening fruit, an increase in nitrate and nitrogen assimilation in the leaves will compete for carbohydrate reserves and can shift the grapevines metabolic priorities from fruit ripening to shoot growth and vegetative vigour (Smart 1985).

1.6.3 Glutamine synthetase and glutamate synthase

The key enzymes involved in the *de novo* synthesis of glutamate in plants are glutamine synthetase-glutamate synthase (GS-GOGAT) (Forde & Lea 2007; Lam et al. 1996). The sequential action of GS and GOGAT enzymes provide the entry of nitrogen in the form of ammonia and its incorporation into amino acid pathways (Figure 1.7).

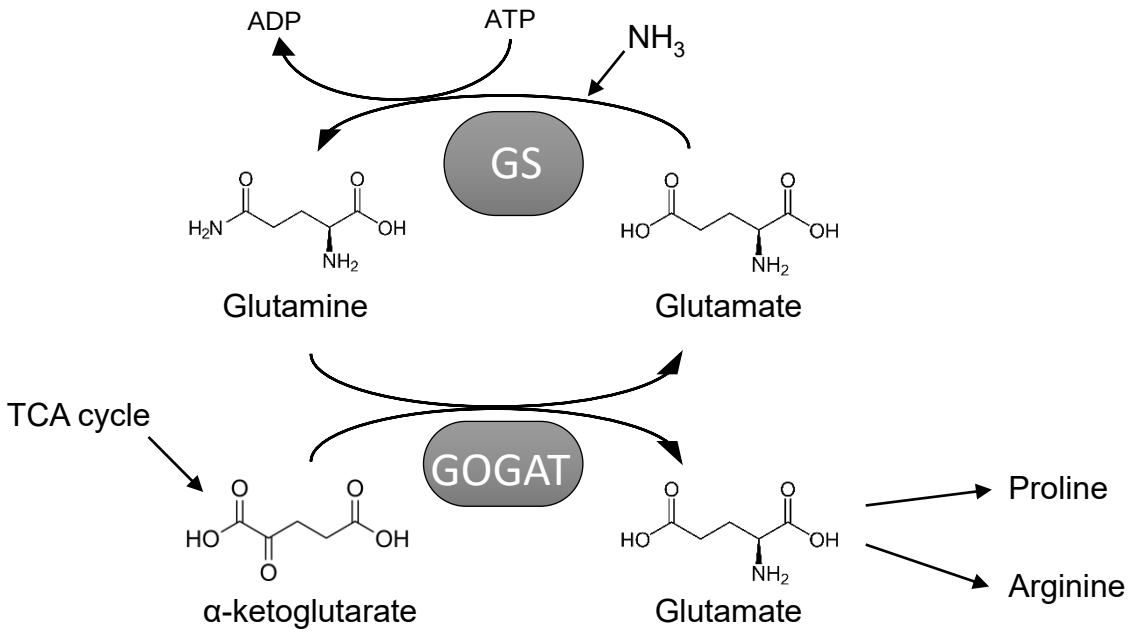


Figure 1.7 Mechanism of nitrogen assimilation in plants.

One of the biochemical pathways incorporating inorganic ammonium into organic amino acids is catalysed by the dual action glutamine synthetase - glutamate synthase (GS-GOGAT) enzymes.

The ATP-dependent synthesis of glutamine from glutamate and ammonia is the first step and is catalysed by GS. GS activity and isoforms can be found in both the cytoplasm and plastids/chloroplasts in most plants, including grapevine (Lam et al. 1996). Subsequently, the GOGAT reaction transfers the amide amino group of glutamine to α-ketoglutarate to yield two molecules of glutamate. The carbon backbone of α-ketoglutarate is provided by the TCA (tricarboxylic acid or Krebs) cycle. One of the molecules of glutamate produced by GOGAT is used to regenerate the GS-GOGAT cycle, "recycled" back for further ammonia assimilation to produce additional glutamine via more rounds of GS and GOGAT action, and so on and so forth. The other glutamate molecule can be used to produce a variety of other amino acids either through the action of aminotransferase enzymes, or in the example of proline and arginine, as a precursor for their biosynthetic pathways (Forde & Lea 2007). Additionally, in the roots,

glutamate can be converted back to glutamine by a different form of GS for export in the xylem to the leaves and shoots. Therefore, glutamine, either directly via aminotransferases, or indirectly via glutamate provide N groups for almost all of the organic nitrogen containing compounds in the grapevine (Keller 2015).

GS activity has been detected in a number of grapevine tissues such as leaves, shoots, roots and berries (Ghisi et al. 1984; Loulakakis & Roubelakis-Angelakis 1996; Roubelakis-Angelakis & Kliewer 1983). The grapevine gene family of GS shows a similar complexity to other plants; with a number of homologous cDNA transcripts and differential expression across different tissues and developmental stages (Loulakakis & Roubelakis-Angelakis 1996, 2000). The GS enzyme proteins exist as multiple isoforms in most higher plants and there are two types; activity of the first type (GS1) is located in the cytoplasm and the other is restricted to chloroplasts/plastids (GS2) (Peterman & Goodman 1991). In growing shoots and leaves, the chloroplast GS2 is considered to be the main isoform responsible for nitrogen assimilation. Whereas the cytosolic GS1 isoforms are encoded by a small family of genes and their expression and enzyme activity varies according to plant species, developmental state and tissue type (Cren & Hirel 1999).

Two forms of GOGAT with separate cofactor specificities have been detected in plants; one using NADH (NADH-GOGAT) as a reductant and the other utilising ferredoxin (Fd-GOGAT). The two forms of the enzyme are structurally distinct differing in their molecular sizes, enzyme kinetics and localisations (Gregerson et al. 1993; Lea et al. 1990; Suzuki et al. 1982). Fd-GOGAT is compartmentalised in chloroplasts and is the main enzyme form in photosynthetically active green tissues, where it can constitute up to 1% of the protein content of leaves (Matoh & Takahashi 1982). The NADH dependent form of GOGAT is localised to plastids and has been described in a range of non-leaf tissues (Gregerson et al. 1993; Suzuki et al. 1982). Activity of both the Fd-GOGAT and NADH-GOGAT forms of the enzyme has been found in grapevine tissues (Creasy & Breen 1997; Loulakakis & Roubelakis-Angelakis 1997).

1.7 Amino acid biochemical pathways - proline metabolism

Proline and arginine are the two amino acids which accumulate to the highest levels in grape berries and their metabolism can also be linked through another amino acid, ornithine. Their importance as nitrogen storage compounds and regulatory molecules are discussed below.

1.7.1 The role of proline in plants

Proline accumulates to high concentrations in many higher plant species in response to a variety of environmental stresses, but often it has been linked to osmotic stress (Csonka and Hanson 1991; Hare and Cress 1997). Because of its high solubility in water, it is considered a compatible solute/osmolyte and is thought to protect against conditions of drought and high salinity (Le Rudulier et al. 1984; Szabados & Savoure 2009; Zhang & Becker 2015). Nevertheless, proline accumulation has also been correlated to a wide range of other abiotic stresses including UV radiation, heavy metals and oxidative stress (Figure 1.8).

NB. A compatible osmolytes primary function is to maintain cell turgor and a positive gradient for water uptake into the cell. There is also evidence for compatible solutes acting as free-radical scavengers or chaperones by chemically interacting with and stabilising proteins (Heuer (2010) and reference within).

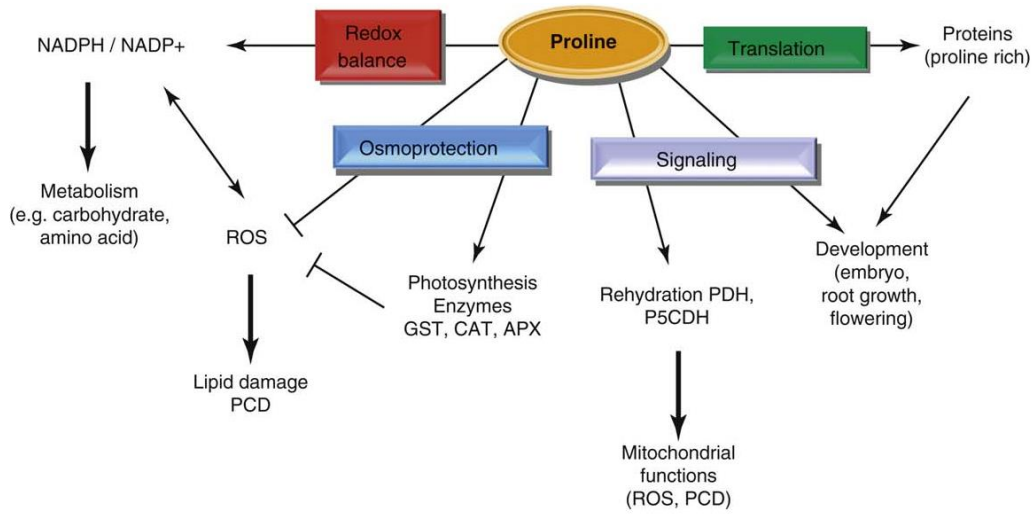


Figure 1.8 The multiple and varied roles of proline in plants.

Proline functions as an osmolyte, is used for protein biosynthesis and is a component of metabolic signalling networks controlling stress and development (from Szabados & Savoure (2009).

The response of the plant to accumulate proline is often triggered by the primary stress signal, for example, ion imbalances caused by high salinity in osmotic stress. Additionally, there are secondary signals induced by the primary response and these can include phytohormones, mainly abscisic acid (ABA), and reactive oxygen species. ABA signalling plays a significant role in plant stress responses and proline accumulation (Abraham et al. 2003; Bensen et al. 1988; Hare et al. 1999; He & Cramer 1996). Whatever the stress signal, accumulated proline can later be used to provide a supply of energy and a store of carbon and nitrogen, catabolised once the stress episode is relieved (Szabados & Savoure 2009).

Far from being a passive molecule, or an "inert" compatible osmolyte, a number of more recent studies with mutants and transgenic plants have shown that proline metabolism also responds to developmental and stress cues (Mattioli et al. 2008; Miller et al. 2009; Szekely et al. 2008). The plants responses to these cues can modulate proline accumulation with intracellular proline levels being maintained through a balance between biosynthesis, catabolism, transport and cellular compartmentalisation. The regulation of proline metabolism and compartmentalisation is covered in detail in Chapter 1.7.4. Therefore, proline accumulation can influence stress mechanisms in a variety of ways, having multiple functions stimulated by both endogenous and external environmental signals (Figure 1.8).

1.7.2 Proline biosynthetic pathways

In plants, the main route of proline biosynthesis is a two-step process using glutamate as a starting precursor (Figure 1.9). An alternative pathway of synthesis is from ornithine, which means that this route of synthesis is interconnected with arginine metabolism. This alternate pathway of biosynthesis via arginine and ornithine is discussed in Chapter 1.9.

The first step in the synthesis of proline is the reduction of glutamate to glutamate-semialdehyde (GSA) by the bi-functional enzyme pyrroline-5-carboxylate synthetase (P5CS). GSA then spontaneously converts to pyrroline-5-carboxylate (P5C) (Hu et al. 1992; Savoure et al. 1995), an intermediate compound in both the biosynthetic and catabolic pathways of proline. Subsequent reduction of the P5C intermediate to proline is catalysed by the enzyme pyrroline-5-carboxylate reductase (P5CR) (Szoke et al. 1992; Verbruggen et al. 1993). Both P5CS and P5CR use NADPH as a cofactor to provide reducing equivalents.

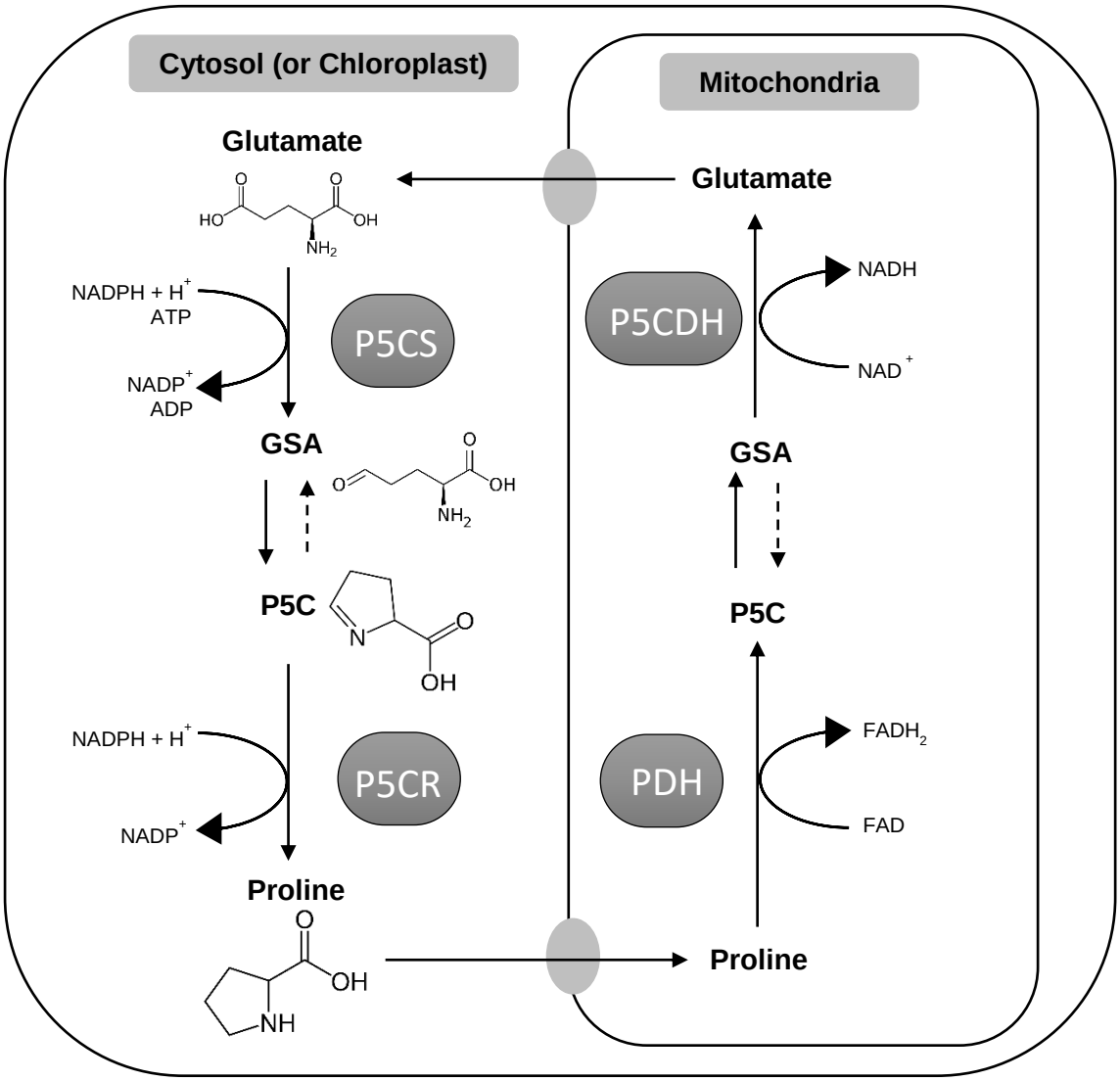


Figure 1.9 The proline metabolic pathways in plants.

The proline metabolic pathways are compartmentalised within the cell. Proline biosynthesis generally occurs in the cytosol, but at times of stress may also be active in chloroplasts. The enzymes for proline degradation are located in the mitochondria. Abbreviations – P5CS: pyrroline-5-carboxylate synthetase; P5CR: pyrroline-5-carboxylate reductase; PDH: proline dehydrogenase and P5CDH: pyrroline-5-carboxylate dehydrogenase.

In many plant species, P5CS is encoded by two genes, one of which is induced by stress. For example in Arabidopsis, general levels of proline in the cytosol are controlled by the *P5CS2* gene, but during periods of osmotic stress, proline biosynthesis is upregulated in the chloroplasts, induced by the stress-regulated *P5CS1* gene (Savoure et al. 1995; Strizhov et al. 1997; Szekely et al. 2008). Other studies confirm that P5CS is the rate-limiting enzyme of the glutamate pathway of proline biosynthesis and the differential expression of *P5CS2* and *P5CS1* genes suggests they have distinct roles to developmental and environmental cues (Kavi Kishor et al. 2005, 1995). P5CR is encoded by one gene and has also been

shown to respond to osmotic stress in some plants examined (Rhodes et al. (1999) and references within), however, P5CR is not considered to be the rate-limiting step of proline biosynthesis in plants.

1.7.3 Proline degradation pathways

Whereas proline biosynthesis occurs mainly in the cytosol, the enzymes for proline catabolism are located in mitochondria. Proline dehydrogenase (PDH) first converts proline back to P5C before the sequential action of pyrroline-5-carboxylate dehydrogenase (P5CDH) converts P5C to glutamate (Deuschle et al. 2001; Huang & Cavalieri 1979; Kiyosue et al. 1996) (Figure 1.9).

The concentration of proline in plant tissues appears to be controlled by the balance between the biosynthetic enzyme P5CS, and the degrading enzymes PDH and P5CDH (Deuschle et al. 2004). During periods of osmotic stress in *Arabidopsis*, proline catabolism coordinates with proline biosynthesis. PDH mRNA levels are kept low and are repressed by dehydration when levels of *P5CS* mRNA are up-regulated (Sharma & Verslues 2010; Verslues et al. 2007). Proline catabolism appears then to be activated during stress relief and this is controlled by both PDH and P5CDH (Verbruggen et al. 1993; Yoshiba et al. 1997). This is a well characterised mechanism of regulation demonstrated in many plant species. Such a concurrent, reciprocal regulation of biosynthesis and degradation indicates that in certain plants during osmotic stress, the response is to tightly regulate proline levels (Nakashima et al. 1998).

1.7.4 Compartmentalisation and regulation of proline metabolism

Proline metabolism is highly compartmentalised in plant cells. Depending on the environmental conditions, proline can be synthesised in distinct subcellular compartments and proline catabolism is kept separate from its biosynthesis. Such compartmentalisation implies extensive and complex regulation of proline metabolism.

With compartmentalisation comes the need for intracellular transport of metabolites or the ability to facilitate movement (active or passive) of metabolites between the cytosol, chloroplasts and mitochondria. This key area of proline metabolism and regulation is not well characterised. While intracellular transporters of proline have not been identified, some candidates have been described. These include three *Arabidopsis* genes that encode putative amino acid transporters and have an appropriate intracellular localisation and substrate affinity for proline (Grallath et al. 2005). Glutamate/proline co-transporters and mitochondrial proline transporters have also been predicted to be involved in proline transport in mitochondria (Lehmann et al. 2010; Szabados & Savoure 2009). Additionally, basic amino acid transporters have been described that can deliver the related compounds arginine and ornithine through mitochondrial membranes (Palmieri et al. 2006).

Aside from the well described regulation of proline metabolism during dehydration and osmotic stress, little is known about other signalling pathways involved in its regulation. While transcriptional regulation of the proline biosynthetic and catabolic genes are better defined, other mechanisms of regulation have also been implicated, including metabolic control, allosteric inhibition, light regulation, epigenetic mechanisms and endogenous hormone signalling (Hu et al. 1992; Misra & Saxena 2009; Zhang et al. 1995). The regulation of P5CS also involves abscisic acid (ABA), epigenetic control and alternative splice variants. In a similar manner to dehydration, salinity and osmotic stress, *P5CS1* expression can be induced by ABA (Abraham et al. 2003; Strizhov et al. 1997). This ABA induction of *P5CS1* appears to be specific and tightly linked to the activation of proline biosynthesis, as ABA doesn't seem to have a role in the regulation of PDH expression (Sharma & Verslues 2010).

1.7.5 Proline metabolism in grapes - the genes for P5CS and PDH are expressed in grape berry tissue.

As discussed in Chapter 1.5, mature grape berries contain high amounts of proline and its pattern of accumulation is non-uniform throughout berry development, the vast majority of proline accumulation occurring post-veraison in the final 4-6 weeks of ripening (Gregan et al. 2012, 2017; Stines et al. 2000). In grapevine, the research areas related to the proline primary metabolic pathways are not well characterised and there is a very limited literature specifically investigating the genes involved in proline metabolism and their relevance to grape biology.

Because proline functions as an osmoregulator and is stress responsive in other plants (Rhodes et al. 1999), in grape berries its accumulation has often been related to the build-up of high levels of sugars during ripening (Lasa et al. 2012; Stines et al. 2000). However, this has proved contentious and prolines role as an environmental stress response or osmolyte in grapes is so far undefined. Overall, the mechanisms of proline biosynthesis during postveraison berry development still needs to be elucidated.

Southern hybridisation analysis has shown *VvP5CS* to be encoded by a single gene in the grapevine genome, with expression studies (Northern analysis) revealing that *VvP5CS* is expressed in a range of grapevine tissues, including leaves and grape berries (Stines et al. 1999). *VvP5CS* mRNA expression in grape berries remained relatively constant throughout their development, aside from transient increases observed at 4 and 12 weeks postflowering. The transient increases in *VvP5CS* expression was not translated into changes in levels of P5CS protein as measured by Western blotting. Investigating post-transcriptional and post-translational control of VvP5CS, Stines et al. (1999) also demonstrated that the P5CS recombinant enzyme was subject to feedback inhibition by proline and the level of inhibition was influenced by glutamate concentration, similar to P5CS from other plants. However, more recent research has detected expression of two *P5CS* isogenes and three proline transporter isogenes, which were up-regulated in ripening berries during proline accumulation (Rienth et al. 2014). This

suggests that other proline genes may be present in grapevine, but this has not been confirmed by any other studies and no further characterisation of these putative isogenes has been investigated.

There is likely to be other points of influence regulating the temporal pattern of proline accumulation in developing grape berries, other than just simple regulation of *VvP5CS* expression and translation, including regulation of the proline catabolism pathway. In grape berries, the proline degrading enzyme PDH protein levels have been shown to increase throughout development to relatively high levels late in berry development, potentially induced by increases in proline (Stines et al. 1999). This high level of PDH protein overlaps with the time of most rapid proline accumulation and indicated that accumulation is not due to a decrease in proline degradation. This observation does not rule out the potential for PDH to be under some compartmentalisation or post-translational control altering PDH activity. In a broad proteomic study investigating the effects of ABA treatment on grape berries, a decrease in P5CDH protein was observed after ABA treatment (Giribaldi et al. 2010). The authors postulate a hypothesis in which proline accumulation in ripening berries may be under ABA control by inactivation of the catabolic enzyme P5CDH, but again, this was not further investigated.

1.8 Amino acid biochemical pathways - arginine metabolism

1.8.1 The role of arginine in plants

In plants, arginine is used as a form of nitrogen storage and a precursor of other biological compounds. Out of the amino acids, arginine has a high nitrogen to carbon ratio (which helps its suitability for storage) and its synthesis is closely regulated by a number of feedback mechanisms which are related to the plants nitrogen status.

Arginine is the precursor to a family of biological molecules called polyamines. Polyamines, which include putrescine, spermidine and spermine are important during plant development, including the growth stages of fruit ripening and leaf senescence. Polyamines are also involved in abiotic stress responses, the regulation of nitrogen assimilation and balancing general nitrogen metabolism in plant cells (Winter et al. (2015) and references within). Additionally in *Arabidopsis*, arginine has been implicated in arginine-dependent nitric oxide (NO) production (Flores et al. 2008; Shi et al. 2013), NO having a wide array of roles in regulating plant growth and development.

There is also a potential link between arginine and proline metabolism via the amino acid ornithine. The most prominent hypothesis is that high levels of arginine could be used to bolster proline accumulation at times of abiotic stress in plants, particularly osmotic stress. However, direct evidence for this mechanism of proline accumulation in plants has yet to be demonstrated.

1.8.2 Arginine biosynthesis via ornithine

The biosynthetic route to arginine synthesis in plants can be divided into two distinct processes; the synthesis of ornithine from glutamate, and then subsequent synthesis of arginine from the ornithine intermediate. Glutamate is therefore a precursor for both arginine and proline biosynthesis in plants (Forde & Lea 2007).

The biosynthesis of ornithine from glutamate is the first part of the pathway (the “cyclic” pathway) and occurs through a number of enzyme catalysed steps and acetylated intermediates and is summarised in Figure 1.10. The first step is the acetylation of glutamate by the enzyme *N*-acetylglutamate synthase (NAGS) to form *N*-acetylglutamate, thus committing glutamate to ornithine synthesis (Slocum 2005). The *N*-acetylglutamate-semialdehyde intermediate several steps along in this pathway, is incapable of cyclising, in contrast to the non-acetylated form of GSA in proline biosynthesis which cyclises spontaneously to form P5C. This means that the acetylation of glutamate by NAGS commits it to the ornithine biosynthetic pathway and excludes it from proline biosynthesis (Caldara et al. 2008). Arginine synthesis from the ornithine intermediate is catalysed by enzymes of the so-called “arginine pathway” (Micallef & Shelp 1989; Slocum 2005). In addition to glutamate being important in the cyclic pathway,

the amino acids aspartate and glutamine are essential cofactors in the “arginine pathway”, by being substrates for ornithine transcarbamylase and argininosuccinate synthase and providing nitrogen atoms to the synthesis of arginine (Figure 1.10).

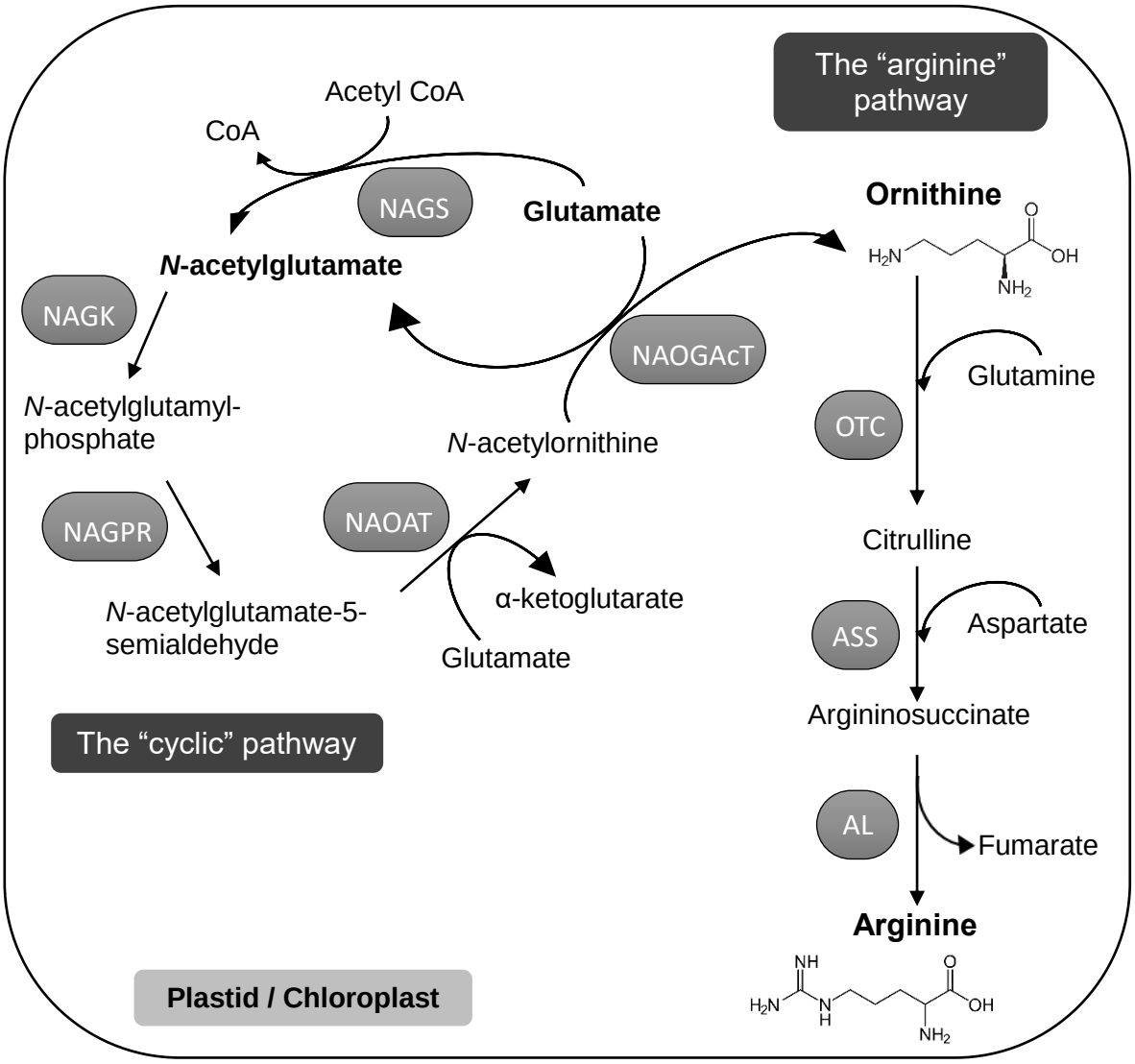


Figure 1.10 The arginine biosynthetic pathway in plants.

Arginine synthesis in plant chloroplasts can be divided into two distinct pathways, the cyclic pathway and the arginine pathway. Upon the formation of ornithine (from N-acetylornithine) leading into the arginine pathway, N-acetylglutamate is regenerated back into the cyclic pathway. Abbreviations - NAGS: N-acetylglutamate synthase; NAGK: N-acetylglutamate kinase; NAGPR: N-acetylglutamatyl-5-P reductase; NAOAT: N-acetylornithine aminotransferase; NAOGAcT: N-acetylornithine-glutamate acetyltransferase; OTC: ornithine transcarbamylase; ASS: argininosuccinate synthase; ASL: argininosuccinate lyase.

1.9 Amino acid biochemical pathways - ornithine metabolism

While the "main" source of proline biosynthesis in plants is through the cytosolic pathway from glutamate via a GSA/P5C intermediate, a potential alternative pathway of proline synthesis is from ornithine, thereby linking it to arginine metabolism (Figure 1.11).

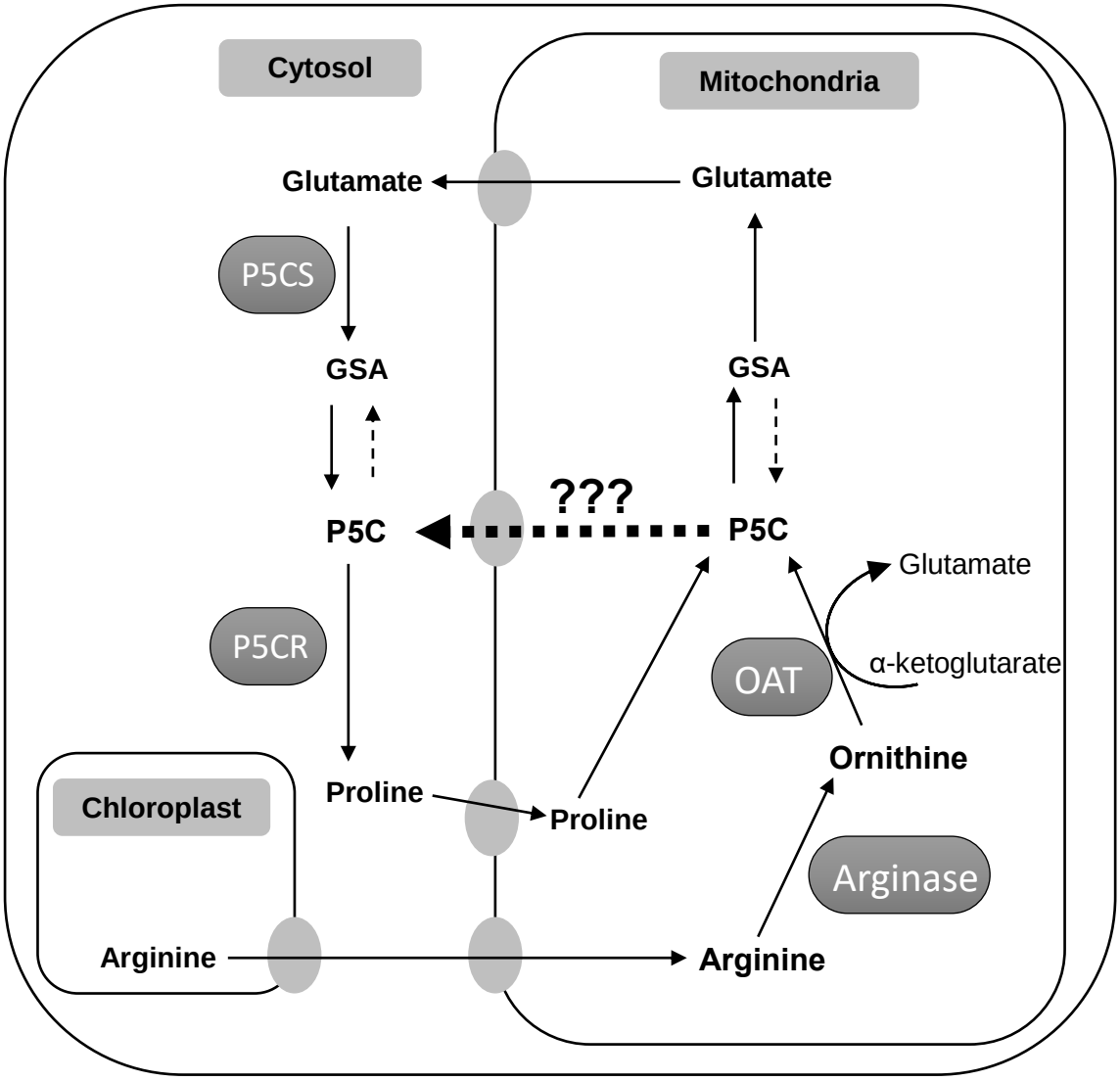


Figure 1.11 Arginine and ornithine metabolism in plants.

Arginine catabolism occurs in the mitochondria by the enzymes arginase and ornithine aminotransferase (OAT), forming the intermediate ornithine and finally P5C. The ability of P5C to directly contribute to proline biosynthesis would require transport out the mitochondria into the cytosol by an as yet unknown mechanism.

Upon import into mitochondria (arginine biosynthesis being localised predominantly in plastids/chloroplasts), arginine catabolism by the enzyme arginase (arginine ureahydrolase) converts arginine to ornithine and urea. Catabolism proceeds by the transamination of the δ-amino group of ornithine to α-ketoglutarate catalysed by the enzyme ornithine aminotransferase (OAT), producing GSA and glutamate. As GSA spontaneously cyclises to P5C, the common intermediate in proline biosynthesis and degradation, it has been postulated that formation of P5C as a direct contribution from ornithine (and the activity of OAT) could boost proline accumulation (Delauney & Verma 1993; Stines et al. 2000).

The model of conversion of ornithine to P5C by OAT, for which it can then serve as a direct substrate for proline synthesis has proven controversial. OAT is localised to mitochondria where no proline synthesis enzymes are located, and therefore would require a mechanism to transport P5C to the cytosol, where P5CR is located (Funck et al. 2008; Stranska et al. 2008). Nevertheless, there is some evidence for this model. Applying exogenous arginine and ornithine to plants has been shown to increase proline accumulation, while supplementation of labelled arginine can be recovered as proline (Adams & Frank 1980; da Rocha et al. 2012). Transport of P5C into and out of the mitochondria as part of a novel P5C-proline cycling pathway has been postulated, but at present, the identity of the P5C exporters have yet to be discovered (Miller et al. 2009).

Currently, the generally accepted hypothesis is that P5C produced by the activity of OAT inside the mitochondria, is subsequently utilised by P5CDH for the production of glutamate and mitochondrial energy generation (Kavi Kishor & Sreenivasulu 2013; Winter et al. 2015). Glutamate can then be exported from the mitochondria into the cytosol which would then allow it to become a precursor for proline biosynthesis, enhancing accumulation via the canonical pathway (Di Martino et al. 2006; Linka & Weber 2005).

1.9.1 Arginine and ornithine metabolism in grapes

Similarly to proline metabolism in grapevine, research into arginine metabolism and the regulation of OAT has been mostly studied in other plants, with limited studies in grapevine investigating the genes regulating these pathways.

Ornithine provides a link between proline and arginine metabolism and early studies suggested that arginine could act as a precursor for some proline accumulation in developing grape berries. Arginase (converts arginine to ornithine) activity has been detected in grape berries (Roubelakis-Angelakis & Kliewer 1981) and ornithine has been shown to be present in grape berry extracts, albeit at significantly lower levels compared to other amino acids (between 0.3-1% of total amino acids) (Stines et al. 2000). It is therefore possible that at the branch point of ornithine, OAT could link proline biosynthesis to arginine catabolism in grape berries.

A single gene encodes OAT in grapevine and *VvOAT* has been found to be expressed at low levels in a number of grapevine tissues, including mature berries (Stines et al. 1999; van Heeswijck et al. 2001). OAT protein and enzyme activity were also detected at low levels, the authors suggesting such low levels may not contribute significantly to proline accumulation (Stines et al. 1999). In more recent research, as part of a transcriptomic study of berry development in a microvine system, a single *OAT* transcript was detected and down-regulated in green berries, suggesting that this pathway may not be important early in berry development (Rienth et al. 2014).

Even with the limited studies in grapevine, it is clear that the genes and enzymes for proline biosynthesis via the arginine and glutamate pathways (which have been characterised in other plants), exist and appear to be active in grape berries. How much the different routes of biosynthesis determine proline concentrations in the berry, remains an important question for further investigation.

1.10 Partitioning of assimilates

The organic compounds produced from photosynthesis, other metabolism and nutrient assimilation (amino acids for example), in general need to be transported from their place of manufacture (sources) to other places of use or storage (sinks). Photosynthetically capable leaves produce carbohydrates, amino acids and other assimilates and transport them to sink organs (via the phloem) for metabolism or storage. Growing shoot tips and newly emerging leaves are initially sinks because they first need to build up their own photosynthetic capability before they can start producing their own metabolites. But once a leaf has reached maturity and is producing enough metabolites for its own use, it can act as a source and export assimilates to various sinks, including developing grape berries. The distribution of exported assimilates to the sink organs is called partitioning. The various organ sinks compete with each other for available assimilates (subject to supply) and the import rate depends on the sinks demand relative to other sinks being supplied on the same vine.

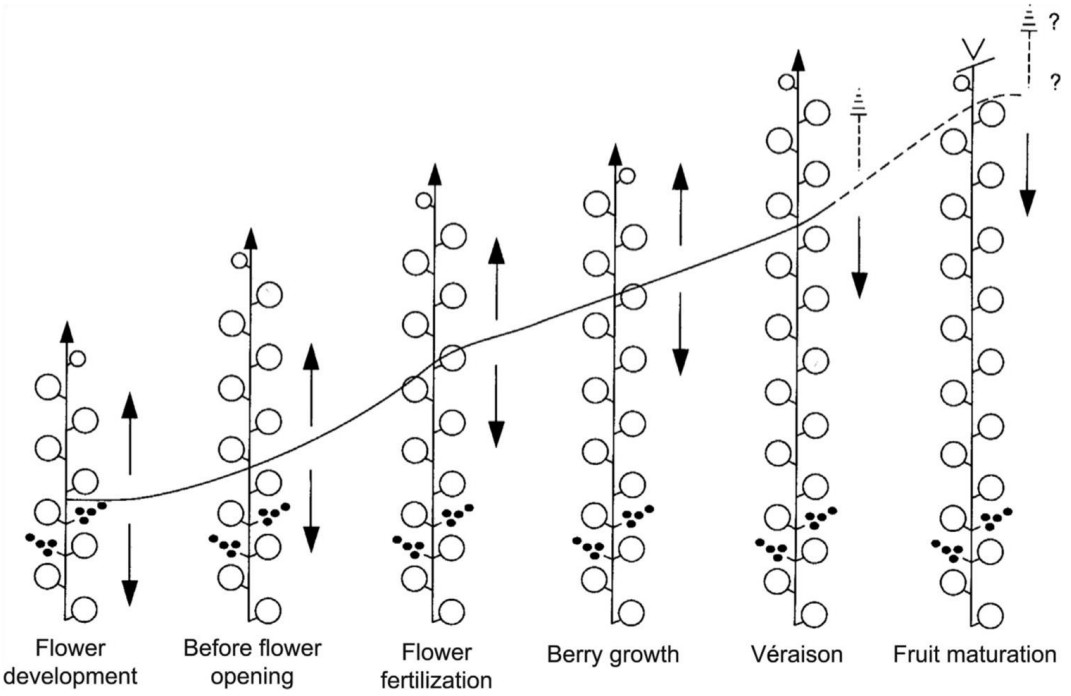


Figure 1.12 Partitioning of assimilates in grapevine.

The source/sink relationship of a growing grapevine shoot changes through development as the shoot lengthens and the grape bunches mature. Arrows indicate direction of transport of assimilates. (taken from Keller (2015)).

A leaf will usually supply assimilates to nearby sinks, but they do not do this equally to all sinks on a plant (Wardlaw 1990). The source/sink relationship of a growing grapevine shoot changes through development as the shoot lengthens and the grape bunches ripen and mature (Figure 1.12). In general, the closer leaves around the grape bunches are more likely to export assimilates to that sink. Mature leaves nearer the shoot tips, supply the extending tip and intermediate leaves can export to sinks in both directions (Hale & Weaver 1962; Koblet et al. 1993). Additionally, many other factors can modulate the relative strength of a sink including competition between sinks, plant communication and signalling, developmental stage and maintenance of vascular connections (Keller (2015) and references within). But in general, for strong sinks it is beneficial to be proximal to a source. The basal leaves therefore preferentially supply the grape bunches, especially as the fruit matures and becomes a strong and dominant sink for assimilates (Wardlaw 1990).

1.10.1 Canopy leaf removal in vineyards

As described in Chapter 1.5, any factor that influences vine growth and metabolism, such as vine nitrogen status, can lead to large variations in fruit quality. Changes to so-called “vine balance” can also be achieved through canopy management practices. The aim of such practices is to regulate vine vigour, size and quality of the grapes and grapevine health. Like vine nitrogen status, manipulation of the vine leaves (including shoots, laterals and overall canopy) can alter source-sink relationships, bunch

microclimate and vegetative growth. This in turn, modifies a multitude of overlapping environmental factors including light, temperature and humidity.

Viticulturists of commercial vineyards routinely subject grapevine canopies to considerable manipulation through pruning, trellising and canopy management. Removing leaves (either by hand or more commonly in New Zealand, by automated approaches) and laterals facilitates opening up the canopy in the fruiting zone and around the grape bunches. This has the effect of reducing humidity around the fruit by letting sunlight (UV radiation) and airflow penetrate the grape bunches, but also allows better spray penetration to the fruit for pest and disease control.

Most commonly in commercial vineyards, leaf removal takes away leaves from the basal portions of shoots where the grape bunches are located. In New Zealand, a variety of leaf removal techniques (often automated) are utilised, and this is generally carried out between flowering and veraison (Creasy & Creasy 2009). On dense canopies, leaf removal has been shown to benefit improved ripening and grape quality and a lessening of disease pressure (Poni et al. (2018) and references within). One of the biggest local effects of leaf removal is an alteration in source-sink balances and a potential loss of photosynthetic capability. Most of a grapevines photosynthesis occurs in the leaves, while photosynthesis in the grapevine shoots and berries is negligible. Nevertheless, depending on the extent of the leaf removal, exposure of the underlying leaf layers and compensation of photosynthetic capacity from other parts of the canopy (for example, newer apical-located shoot leaves) means that the effects of basal leaf removal are generally not detrimental to fruit development.

1.10.2 Partitioning of assimilates - not every leaf is equal

One of the most pronounced effects of leaf removal is the induction of anthocyanin and flavonol biosynthesis, due to the increased light exposure of the grape clusters (Dokoozlian & Kliewer 1996; Haselgrove et al. 2000; Liu et al. 2015; Smart 1985). But aside from the more obvious effects of light exposure due to leaf removal, canopy management techniques such as leaf removal at different developmental stages or targeting distinct regions of the canopy and lateral thinning can markedly change grape composition (total soluble solids, amino acids and aromatic compounds, for example) and wine sensory properties (Palliotti et al. 2011; Poni et al. 2006).

Partitioning influences presumably have a significant role in these observations from previous studies. Any source modification, such as basal leaf removal for example, will force the more distal leaves on the shoot to export a greater proportion of their assimilates to the clusters to compensate for this loss (Quinlan & Weaver 1970). Additionally, a large number of studies using leaf removal as an experimental tool to target specific leaves, have shown that some leaves are more dominant than others for supplying certain assimilates into the grape berry. For example, an important compound in grapes, 3-isobutyl-2-

methoxypyrazine, has been detected in both leaves and stems (Hashizume & Umeda 2014; Roujou de Boubee et al. 2002) and the authors found much higher concentrations in the basal leaves proximal to the grape bunches, compared to more apical leaves further up the shoot. Furthermore, targeting different regions of the leaf canopy (basal vs apical leaf removal for example) has also demonstrated differential effects on TSS accumulation and aromatic compounds concentrations in the grapes (Poni et al. 2018).

1.10.3 Basal leaf removal affects amino acid accumulation in Sauvignon blanc grapes

Our research group has previously shown that leaf removal has a significant effect on amino acid composition in developing Sauvignon blanc grapes, significantly decreasing total amino acid concentrations (Gregan et al. 2012). This investigation of amino acid accumulation in response to leaf removal and ultraviolet (UV) light modification, was part of a broader study examining Sauvignon blanc berry composition (monitoring amino acids, flavonols and methoxypyrazines). While the impact of leaf removal clearly decreased amino acid accumulation, the amino acid composition showed little consistent differences in response to UV-B radiation exclusion. To confirm the validity of the experiment and interpretation of results, exposure of the fruiting zone to UV radiation by leaf removal had a profound influence of flavonol levels. The effects of increasing flavonol levels were mediated through the UV-B part of the light spectrum (Gregan et al. 2012; Liu et al. 2015).

Even though this study was limited in sampling time points, the results nevertheless suggested that the maintenance of a leaf canopy around the grape bunches is important to support levels of amino acids in the grapes and indicated the potential for further research in this area.

1.11 Hypotheses and summary

The aim of this thesis is to improve our understanding of the mechanisms influencing amino acid biochemistry in *Vitis vinifera* L. var. Sauvignon blanc grapes.

The overall hypothesis that I am testing is that: qualitative and quantitative changes to amino acid composition in response to basal leaf removal in Sauvignon blanc grapes, are mediated through partitioning and source/sink modifications and changes in gene expression of associated amino acid metabolic and regulatory pathways.

The above hypothesis was tested by studying the effects of basal leaf removal performed at two developmental stages during the two subsequent 2013 and 2014 growing seasons. Leaf removal was carried out at preveraison and postveraison developmental stages and a comprehensive sampling regime was undertaken, collecting berry samples at multiple timepoints during development. A third vineyard experiment was utilised during the 2018 growing season to provide fresh material for biochemical enzyme assays.

The objectives for this study were:

1. To develop an understanding of the contribution that vineyard canopy management (in the form of basal leaf removal) has in determining the amino acid concentrations in the grape berry.
    - How basal leaf removal around the fruiting zone of the vine influences amino acid accumulation in the berries.
    - How basal leaf removal around the fruiting zone of the vine influences qualitative aspects of the proportions of individual amino acids in the berries.
2. To examine the mechanisms of amino acid accumulation by investigating a number of genes involved in different aspects of amino acid biochemistry, including assimilation, biosynthesis, catabolism and regulation. The focus was regulation of the α-ketoglutarate family of amino acids (glutamine, glutamate, arginine and proline), being the family that contains the predominant concentrations of amino acids in the berry.
    - Are genes involved in the metabolic pathways of the α-ketoglutarate amino acids, differentially expressed through development and in response to leaf removal treatments?

3. To explore a potentially active biosynthetic pathway linking arginine to proline metabolism (via an ornithine intermediate) and determine if stimulation of this pathway could contribute to support proline accumulation.

    - Are genes involved in this alternative metabolic pathway to proline from arginine and ornithine precursors, differentially expressed through development and in response to leaf removal treatments?

    - Can OAT protein enzyme activity can be detected in Sauvignon blanc grapes through development.

While there is extensive research available regarding the role of leaves for berry composition and viticultural efforts to manage/manipulate these factors, much of the data is conflicting and speculative. There is little empirical research investigating how the leaf canopy contributes to determining the amino acid composition of the berry and more specifically, elucidating the mechanisms of amino acid accumulation and related biochemical pathways in the grape berry. Highlighted below are gaps in the literature that will be addressed by this thesis to improve our understanding of grapevine biochemistry.

- Generally, most research investigating amino acid composition in grapes are limited in sampling time points. For example, our previous research was in this area and monitored amino acids in Sauvignon blanc grapes, but this research not specific for amino acids and was part of a broader study examining berry composition (see Chapter 1.10.3). While these results were obtained from three seasons of samples, in two of the seasons, samples were collected at harvest only, and in the other season, just three sampling time points were taken.

- Findings from this thesis will place a greater emphasis on multiple sampling time points, with the aim of following the accumulation of amino acids through development to better define developmental stages where the proportion of individual amino acids is altered.

- The timing of leaf removal will be investigated. Performing both preveraison and postveraison leaf removal experiments will identify how amino acid accumulation is affected by basal leaf removal at different developmental stages.

- In grapevine, the studies investigating proline, arginine and ornithine metabolism are limited and overall, results have formed an ambiguous picture of regulation. The last major study into the genes regulating these pathways specifically in grapevine, was almost two decades ago. A focus of this thesis is to investigate the genetic pathways of nitrogen assimilation and amino acid metabolism in developing Sauvignon blanc berries.

- Although many studies link proline accumulation in plants to environmental stresses, the correlation between proline levels and stress tolerance is not always apparent, and in grape, even less so due to the majority of research being performed in other model plants such as Arabidopsis. The research presented in this thesis is from experiments performed exclusively using grape berry tissue and so will allow for an improved understanding of regulation of proline and arginine metabolism in grapevine.

- Even with the limited studies in grapevine, the genes for proline biosynthesis via the defined glutamate pathway and the arginine/ornithine metabolic genes (arginase and *OAT*), do exist and appear to be active in grapes. While these genes have been well characterised in other plants, an aim of this thesis is to characterise expression of associated genes in grapevine (berry tissue) and perform OAT enzyme assays to investigate the link between arginine and proline metabolism.

Chapter 2
Materials and Methods

2.1 Vineyard experiments

Experiments were carried out in the Lincoln University research vineyard during three seasons (2013, 2014 and 2018). The vineyard is located on the Lincoln University campus (43°38'48'' south, 172°27'29'' east), approximately 30 kilometres from Christchurch in the South Island of New Zealand. The grape vines used for experiments were spread across three rows in the vineyard and represent a mass selected clone of the white grape *Vitis vinifera* L. var. Sauvignon blanc on a SO4 rootstock. The vine rows in the vineyard were planted in a north to south orientation. All experiments described in this thesis were performed exclusively using grape berries from these vines. The Lincoln University vineyard was managed to best industry practice (http://www.nzwine.com/sustainability/sustainable-winegrowing-new-zealand) and also comprised a comprehensive spray regime for disease mitigation. In addition to the experimental treatments detailed below, other canopy management strategies were utilised to control excess vegetative growth of the grapevines and included removal of the growing shoot tips (called “topping”) and side laterals.

2.1.1 Experimental treatments

Three rows of Sauvignon blanc vines were available for experiments, the rows being spaced 2.25 m apart and 1.8 m between vines within the rows. Vines were cane-pruned, retaining three or four canes of approximately 10-12 nodes each. The lower two canes were lightly wrapped to a fruiting wire at 0.9 m and the third/fourth upper canes to a wire at 1.1 m from ground level. Growing shoots were trained in a vertical shoot position using guide wires, which formed a canopy approximately 2 m tall and 0.5 m wide.

Each experimental treatment comprised a block of four vines and was replicated three times. The position of treatment blocks was randomised within the rows. Control treatments (CANOPY) were essentially untouched vines with a complete maintenance of all canopy leaves. The majority of grape bunches in the CANOPY control treatments were in the full shade of the leaf canopy. Leaf removal treatments were equivalent to those used in previous experiments and involved removing leaves from the basal 60 cm of the growing shoots to remove the proximal sink tissues, which subsequently had the effect of fully exposing the grape bunches to sunlight (Figure 2.1) (Gregan et al. 2012; Gregan & Jordan 2016; Liu et al. 2015). Therefore, while 100% of leaves were removed from the basal 60 cm of the canopy (around the grape bunches), significantly 60-70% of the total canopy leaves were still retained above the fruiting zone.

Preveraison leaf removal treatments were applied to the vines with respect to the onset of veraison, taking into account the accompanying berry phenology. The date of veraison and berry phenology also dictated the timing of the postveraison leaf removal treatment. In 2013, two leaf removal treatments were applied to the vines; a preveraison leaf removal (PRE) at -18 DPV (days postveraison) with berry phenology at an average berry weight of 0.76 g and total soluble solids (TSS) of 4.7°Brix; and a postveraison leaf removal (POST) at 6 DPV (average berry weight 1.3 g, 11.0°Brix). In 2014, two equivalent leaf removal treatments were applied to vines; a preveraison leaf removal (PRE) at -23 DPV (average berry weight 0.71 g, 4.1°Brix); and a postveraison leaf removal (POST) at 12 DPV (average berry weight 1.4 g, 12.8°Brix).

A third experiment was also utilised in 2018 to supply fresh grape berry samples for enzyme assays (see Chapter 6). In 2018, a single preveraison leaf removal treatment (PRE) was applied at -27 DPV with berry phenology at an average berry weight of 0.83 g and TSS of 4.5°Brix.

Figure 2.1 Vineyard experiments on Sauvignon blanc grapevines at Lincoln University.

Experiments in 2013 showing; (A) set-up of the preveraison leaf removal (PRE) treatments across a block of Sauvignon blanc vines, and (B) preveraison leaf removal treatments compared to (CANOPY) control vines (far left of photo) showing a complete maintenance of leaves around the grape bunches.

2.2 Sample collection

All samples were taken in triplicate, one from each of the replicated treatments, with 15-40 berries randomly selected per replicate depending on the developmental stage. Berries were sampled from both sides of the vine with no more than one berry taken per bunch. This regime supplied enough berries to give a good representation of metabolite levels, while not removing too many berries and risking unwanted effects such as reducing crop loads. Samples were immediately frozen in liquid nitrogen in the field and stored at -80°C prior to further processing for the appropriate analysis.

In 2013, whole berry samples were collected at 17 time points through development; -18, -8, -1, 6, 8, 10, 13, 15, 17, 20, 24, 27, 30, 37, 44, 51 and 62 DPV. In 2014, whole berry samples were collected at 14 time points through development; -23, -16, -9, -2, 5, 8, 12, 16, 19, 23, 26, 29, 33 and 40 DPV. To monitor berry phenology in 2018, whole berry samples were collected at 13 time points through development; -27, -20, -13, -6, 1, 8, 15, 22, 29, 36, 43, 50 and 57 DPV. Additionally in 2018, fresh berry samples were collected for enzyme assays as described in Chapter 2.8.

2.3 Monitoring developmental parameters

2.3.1 Total soluble solids (TSS) and berry weight

TSS and berry weight were measured throughout the course of the vineyard experiments to monitor grape development. TSS was measured as degrees Brix (°Brix) using a PAL-1 digital hand-held "pocket" refractometer (Atago, Tokyo, Japan). A TSS concentration of 8°Brix was used as a measure of veraison to standardize treatments and results between seasons, veraison dates being on day of the year (DOY) 57 in 2013, DOY 54 in 2014, and DOY 44 in 2018 (Table 3.1). Berry weights were determined by weighing the entire collected sample and averaging the result by the number of berries at that sampling time point.

2.3.2 Accumulated growing degree days (GDD)

GDD counts the total number of degrees Celsius a measured day is above a threshold temperature, in this study, 10°C. As a measure of temperature, GDD can illustrate short-term changes to climatic variations when compared to long-term average climate. The local temperature data used for calculating GDDs was obtained from the Lincoln Broadfield weather station (http://www.cliflo.niwa.co.nz), located approximately 4 km from the Lincoln University vineyard. This station is used routinely by Lincoln University staff to assess weather conditions and to access historical data. Given the flat terrain around the Lincoln district, it provides an accurate measure of temperature and other climatic data in the vicinity of the Lincoln University vineyard.

GDDs were determined using a base temperature of 10°C (Tb=10°C) and were calculated using the average of daily minimum and maximum temperatures minus 10, where no negative values are considered (Winkler et al. 1974). GDDs were accumulated on a daily basis starting on July 1 (southern hemisphere winter) and finishing on June 30 the following year. GDD differences from the long-term average (LTA) were calculated using the GDD on any given day minus the GDD for the LTA. The LTA is the average GDD calculated daily over the past 84 years (1930−2014).

2.3.3 Temperature microloggers

To assess differences in the temperatures around the grape bunches between the CANOPY control and leaf removal treatments, temperature microloggers were used in the 2018 vineyard experiment. Microloggers were placed alongside the grape bunches, hanging off canes that were either naturally shaded by the leaf foliage in the CANOPY control treatments, or fully exposed (and sometimes in the direct sunlight) alongside exposed bunches in the PRE treatment (Figure 2.2). Twelve microloggers (2 for each of 3 replicates x 2 treatments) recorded the temperature every 15 mins, 24 hours a day for the entire duration of the 2018 experiment.

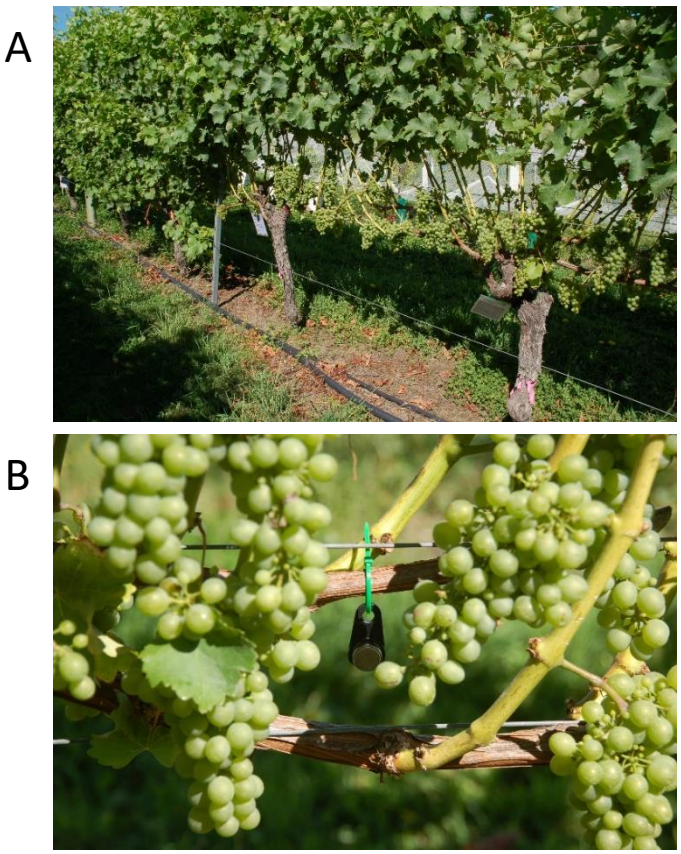


Figure 2.2 2018 vineyard experiments at Lincoln University.

Experiments in 2018 showing; (A) set-up of the preveraison leaf removal (PRE) treatments compared to (CANOPY) control vines (far left of photo) showing a complete maintenance of leaves around the grape bunches, and (B) close up of PRE vines showing temperature microloggers hanging from canes amongst the grape bunches in the PRE treatment.

2.4 Sample preparation for analyses

Whole frozen berries were ground to a fine powder in liquid nitrogen using an IKA A11 Basic Analytical Mill (Merck KGaA, Darmstadt, Germany). The entire sample of berries from each time point was ground using the IKA mill. It was important to use enough berries as to give a representative sample from each treatment (replicate) and a consistent representation of metabolite levels. The high speed IKA mill delivers an impact grinding of tissue using a blunt steel "blade", pulversing all subsets (pulp, skins and seeds) of frozen grape berry tissue to a fine homogeneous powder.

The frozen ground tissue was stored at -80°C prior to further processing for the appropriate analysis. Storing at -80°C and working with liquid nitrogen keeps the ground tissue "running freely", while being easy to weigh and subsample accurately and with consistency.

2.5 Amino acid analysis

Analysis of 20 proteinogenic amino acids (alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine) from the whole berry samples were analysed using a Hewlett-Packard Agilent 1100 series High Performance Liquid Chromatography (HPLC) system (Waldbronn, Germany) as previously described (Gregan et al. 2012).

A subsample (approximately 2-3 g) of frozen grape powder (as processed in Chapter 2.4) was measured into a 15 mL centrifuge tube, allowed to thaw slowly in ice-water for about 20 min and then centrifuged briefly to pellet the solid material. The free-run juice (supernatant) was transferred to a new 1.5 mL microcentrifuge tube and was diluted 1:4 with distilled water. An internal standard (α-amino butyric acid) was added to a final concentration of 100 mmol/L. Depending on the developmental stage and/or amino acid, a number of samples required a higher dilution ratio than 1:4 to allow accurate quantification of certain amino acids with high concentrations. This was reassessed after each run of samples, and the appropriate samples were re-run with greater dilutions on a case by case basis.

The sample was filtered through a 0.45 µm nylon filter into an HPLC glass vial and capped tightly. Each sample was analysed using the above HPLC system with a 250 x 4.6 mm, 5 mm prodigy column (Phenomenex, CA, USA). To derivatise the primary amino acids, *o*-phthaldialdehyde was used as a fluorescence derivative. Iodoacetic and mercaptopropionic acids were used to increase cysteine sensitivity and 9-fluorenylmethyl chloroformate was a fluorescence derivative for proline. Detection utilised a fluorescence detector with an excitation of 335 nm and emission of 440 nm. At 25 min, the detector was switched to a second channel (excitation 260 nm, emission 315 nm) to detect proline. Amino acid standards of known concentrations were analysed in parallel to generate calibration curves for quantification of the unknown samples. The separation used solvent A (0.01 mol/L Na_2HPO_4 with

0.8% THF, adjusted to pH 7.5 with H_3PO_4) and solvent B (20% solvent A, 40% methanol, 40% acetonitrile) with the following gradient: 0 min, 0% B; 14 min, 40% B; 22 min, 55% B; 27 min, 100% B; 35 min, 100% B; 36 min, 0% B; with a flow rate of 1 mL/min. The raw data was analysed using the Chemstation chromatography data system (Agilent, CA, USA).

2.6 Molecular methods

2.6.1 Total RNA isolation

Total RNA for quantitative real-time PCR (qPCR) and Nanostring nCounter analysis was extracted from whole berry tissue using the Spectrum Plant Total RNA kit (Sigma-Aldrich, MO, USA). Approximately 150 mg of frozen powdered tissue (as processed in Chapter 2.4) was transferred to a 2 mL microcentrifuge tube containing 800 µL of lysis buffer (+ β-mercaptoethanol, 1% v/v) and further disrupted using a TissueLyser II (Qiagen, Valencia, CA, USA) before continuing the RNA extraction protocol according to the manufacturer's instructions (with some minor modifications). The sample was then incubated at 56°C for 4 min, followed by centrifugation for 3 min at 14000 x g to pellet cellular debris. Being careful not to disturb the pellet, the supernatant was transferred into a filtration column placed in a 2 mL collection tube and centrifuged at 14000 x g for 1 min to remove residual debris. 750 µL of binding solution was added to the filtered supernatant and mixed immediately with a pipette. The supernatant solution was transferred with a maximum volume of 700 µL into a binding column placed in a 2 mL collection tube and centrifuged at 14000 x g for 1 min. Discarding the flowthrough after centrifugation, this step was repeated to bind all of the supernatant solution. The column now containing bound RNA was washed by adding 500 µL of wash solution-1 and centrifuged at 14000 x g for 1 min. This was followed by two subsequent wash steps (500 µL of wash solution-2) and subsequent centrifugations. The flowthrough was discarded at each step. To fully dry the column and remove any residual wash solution, the column was centrifuged at 14000 x g for 1 min and then transferred to a new 1.5 mL microcentrifuge tube. The RNA was then eluted from the column with 50 µL of elution buffer and centrifugation at 14000 x g for 1 min.

2.6.2 Total RNA purification - DNase treatment

Total RNA samples were treated using the TURBO DNA-free kit (Thermo Fisher Scientific, MA, USA) to remove any potential contamination of genomic DNA. Samples were treated according to manufacturer's instructions with some minor modifications. To the 50 µL RNA sample, 5 µL of 10x Turbo buffer and 1 µL of TURBO DNase enzyme were added, mixed gently by pipetting and incubated at 37°C for 30 min. After incubation, 6 µL of DNase Inactivation Reagent was added to the reaction, mixed and incubated at room temperature for 5 min, mixing occasionally by tapping the tube. The sample was then

centrifuged at 10000 x g for 90 sec and 50 µL of the supernatant (purified RNA) was transferred to a sterile 1.5 mL microcentrifuge tube. The purified total RNA samples were stored at -80°C.

2.6.3 Purified RNA quantification

RNA quantity and quality was determined by using the Qubit 1.0 fluorometer (Thermo Fisher Scientific) and a DS-11 spectrophotometer (DeNovix, DE, USA). The Qubit 1.0 fluorometer was used to quantify the RNA concentrations using the Qubit RNA BR Assay Kit according to the manufacturer's instructions. Additionally, before continuing with further analyses, the DS-11 spectrophotometer was used to calculate the 260/280 nm ratio to estimate the purity of the RNA. A 260/280 ratio of approximately 2.0 is generally accepted as “pure” for RNA. If the ratio is appreciably lower, it can indicate the presence of protein or other contaminants that absorb strongly at or near 280 nm.

2.6.4 cDNA synthesis

cDNA was synthesised using the Primescript RT reagent kit (Perfect Real Time) (Takara Bio, Kyoto, Japan) according to the manufacturer's instructions. For each reaction, 300 ng of RNA was added with 2 µL of 5x Primescript buffer (for a final concentration, 1x), 0.5 µL of Primescript enzyme, 0.5 µL of oligo-dT primer (final concentration, 25 pmol) and 0.5 µL of Random-6mer primers (final concentration, 50 pmol), adjusting with RNase-free water to a final volume of 10 µL. The reaction was incubated at 37°C for 15 min for reverse transcription, and then heated at 85°C for 10 sec to inactivate the enzyme. cDNA for qPCR was diluted 1:25 in distilled water.

2.6.5 Quantitative real-time PCR (qPCR)

Expression of *VvFLS4*, *VvGAPDH*, *VvActin*, *VvEF-1α* and *VvSAND* genes were analysed by qPCR using the Eco Real-Time PCR System and Eco/EcoStudy software v4.0 (Illumina, CA, USA). Each reaction was prepared in 15 µL, which consisted of 7.5 µL of SYBR Premix Ex TaqII (Takara Bio), 0.2 µmol/L of each (forward and reverse) primer, 5 µL of diluted template (cDNA or standards), and the appropriate volume of distilled water. Negative controls, which contained distilled water substituted for template, were included in each run. Samples were run in duplicate. To ensure consistency and reproducibility, qPCR reactions were set up using an epMotion 5070 robot controlled by the epBlue software system (Eppendorf, Hamburg, Germany). The thermal cycling conditions were an initial denaturation at 95°C (30 sec) for polymerase activation, followed by 40 cycles of 95 °C for 10 sec, 56 °C for 30 sec, and 72 °C for 30 sec.

All the primers (Appendix 1) used for qPCR analysis in this study were specific and amplified products with the expected size and correct sequence, which was confirmed by DNA sequencing and melting curve analysis after qPCR amplification (Gregan & Jordan 2016; Liu et al. 2015) (data not shown). The

primers for VvFLS4 were taken from a previous study (Downey et al. 2003). The primers for the reference genes were either designed using the Primer3Plus software (Untergasser et al. 2012) or taken from a previous study (Reid et al. 2006). Vector constructs were generated for use as qPCR standards as previously described (Gregan & Jordan 2016; Liu et al. 2015). The standards were made by cloning partial cDNA fragments into the pGEM-T Easy Vector System (Promega, WI, USA), then transforming into One Shot TOP10 Chemically Competent Escherichia coli cells (Thermo Fisher Scientific), and purifying using an ISOLATE II Plasmid Mini kit (Bioline, London, UK), all according to the manufacturer's instructions.

Relative quantitation of *VvFLS4* expression was performed using the standard curve method, normalized to the internal reference genes *VvGAPDH* and *VvActin* (see Chapter 2.6.6). Standard curves were generated using serial dilutions of vector constructs (containing target gene fragment) of known concentrations (10^{-2}, 10^{-4} and 10^{-6} ng/µL). Relative quantitation was obtained by graphing the cycle threshold (Ct) values against the standard curves and then normalizing to the average values for both reference genes in each respective sample.

2.6.6 Determination of reference (housekeeping) gene stability for qPCR and nCounter analysis

The reference genes *VvGAPDH*, *VvActin*, *VvEF-1α* and *VvSAND* were all initially analysed for their expression stability through development using the RNA/cDNA samples from the 2013 and 2014 vineyard experiments. These four reference genes had previously been identified for their relative stability in developmental real-time PCR studies of grape berry (Reid et al. 2006). For each gene, obtained Ct values from qPCR reactions (set up as in Chapter 2.6.5) were converted into relative quantities for analysis with the geNorm v3.4 software (Vandesompele et al. 2002). This program calculates an expression stability measure for each gene, whereby it then performs a stepwise exclusion of the least stable gene until the two most stable genes are left. In our geNorm analysis, *GAPDH* and *Actin* remained as the two most stably expressed genes in berry samples across the 2013 and 2014 experiments. Therefore, for qPCR and nCounter analyses (described below), *GAPDH* and *Actin* were used as the reference genes for normalisation of data.

2.6.7 Bioanalyzer analysis

The quality of a "representative" subset of 24 RNA samples that were to be sent for nCounter analysis, were further analysed using an Agilent 2100 Bioanalyzer. The samples chosen were spread across the developmental stages from the 2013 and 2014 vineyards experiments. An RNA Nano 6000 kit (Agilent) was used to prepare each "RNA chip" as per the manufacturer's instructions. 5 µl of RNA 6000 Nano marker was added to all wells, before 1 µl of sample was added to each sample well. 1 µl of ladder was

added to the ladder well. The chip was run on the 2100 Bioanalyzer and analysed with the accompanying software. RNA Integrity Number (RIN) values and gel images were generated within the Bioanalyzer software.

2.7 Nanostring nCounter analysis

Nanostring nCounter experiments were performed offsite, services being provided by New Zealand Genomics Limited (NZGL) in partnership with Otago Genomics Bioinformatics Facility (Dunedin, New Zealand). Purified total RNA samples were forwarded to NZGL for nCounter analysis. The samples were sent on dry ice to preserve RNA integrity and stored at -80°C upon receipt.

2.7.1 Fragment Analyzer analysis

NZGL first performed their own RNA quality control before continuing with any analyses. All 198 samples were fragment analysed using a Fragment Analyzer system (Agilent), according to NZGL protocols.

2.7.2 nCounter transcript analysis

All RNA samples were processed by NZGL using their standard nCounter analysis protocols. Samples were processed in batches of 12 using a proprietary Prep Station robot (Geiss et al. 2008). Each reaction consists of (multiple) pairs of reporter and capture probes that hybridise to the target sequences of interest (Appendix 2), forming a tripartite complex that can be measured by a digital image analyser and quantified into “raw transcript counts”. In addition to the genes being targeted for quantification of transcript counts, reactions also contained *GAPDH* and *Actin* reference gene probes for data normalisation. Also present in the reactions are six pairs of positive-control and two pairs of negative-control probes (reporter and capture), which are used to standardise the data for any differences in reaction efficiencies (Geiss et al. 2008). Raw data generated from nCounter analysis was exported as proprietary RCC files to be analysed using NanoString’s nSolver data analysis software.

2.7.3 nCounter data analysis

The RCC files obtained by nCounter analysis were imported into the nSolver data analysis software and used to extract the raw transcript counts from all genes analysed. Normalisation of transcript counts was performed at Lincoln University and were generated manually following a designated workflow in three steps:

1) Multiplying all raw counts by a scaling factor calculated from the geometric means of the positive control counts.

2) Following step 1, subtracting the geometric means of the negative control counts to remove any background effect.

3) Finally, to calculate the normalised counts, multiply all background corrected counts by a normalisation factor calculated from the geometric means of the reference genes.

Following this workflow eliminates variability unrelated to the sample itself (using positive and negative assay controls,) before adjusting the transcript counts relative to the reference genes, producing the normalised transcript counts. It is these normalised transcript counts that are presented for expression analysis in Chapter 5.

2.8 OAT enzyme assays

The assay procedure used for analysing OAT activity in Sauvignon blanc grapes was a variation on the methods reported by Kim et al. (1994) and Funck et al. (2008). Fresh whole grape berry samples were rinsed in sterile water, cut into small pieces and ground using a mortar and pestle in ice-cold assay buffer (100 mmol/L potassium phosphate buffer, 1 mmol/L EDTA, 0.2 mmol/L pyridoxal phosphate, pH 7.9). For each replicate analysed, six grapes were ground with 4 mL assay buffer. The extract was centrifuged for 30 sec at 0.1 rcf (4°C) to pellet and remove excess cellular material and debris. The resulting supernatant was the enzyme extract and used for the OAT activity assays and determination of total protein content.

A typical assay was performed in 500 uL volumes. The assay mixture contained 25 mmol/L ornithine, 25 mmol/L α- ketoglutarate, 100 µL or 200 µL enzyme extract, and finally adjusted to a total volume of 500 µL with assay buffer. In addition, reaction controls were included in each assay to provide background absorbance measurements and processed identically as the activity assays. Each reaction was incubated for 20 min at 37°C and then terminated by the addition of 150 µL of 3 M perchloric acid. The target enzymatic product of P5C was detected by adding 100 µL of 2% ninhydrin and heating the reaction to ≈100°C for 5 min in a heating block. After heating, reactions were placed immediately on ice for 10 min. The water-insoluble reddish precipitate that formed was centrifuged at 14000 x g for 3 min and dissolved in 1 mL of ethanol. Absorbance was measured at 510 nm using a FLUOstar omega microplate reader (BMG Labtech, Ortenberg, Germany). For Time 0 (min) baseline readings, reactions were set-up as above, but then terminated immediately with perchloric acid and subsequently processed as normal.

Any variations to the above procedure that were used for optimising and troubleshooting the OAT activity assay are described in detail in Chapter 6.3.

2.8.1 Determination of total protein content of enzyme extracts

To generate a measure of specific enzyme activity based on the protein content of enzyme extracts, the protein concentration of extracts was determined using the Bradfords protein assay. Standard curves were generated using known concentrations (serial dilutions of 0-1000 µg/mL) of bovine serum albumin (Sigma-Aldrich, MO, USA) in enzyme assay buffer. Standard curve samples (10 µL) and "unknown" extract samples (10 µL) were transferred to a microplate. After the addition of 200 µL of Bradford reagent (BioRad, CA, USA), the microplate was incubated at room temperature for 5 min and then the absorbance was measured at 595 nm using a FLUOstar omega microplate reader. The absorbance of the "unknown" extract samples was compared to the generated standard curves and used to determine their concentrations.

2.9 Statistics

All statistical analyses were conducted using Microsoft Excel and the GenStat statistical software package (VSN International, Hemel Hempstead, UK). Means and standard errors of the mean (SEM) were calculated combining replicates of measurements at each time point. All data was analysed using analysis of variance (ANOVA) and a Fisher's least significant difference (lsd) test. A *p*-value less than 0.05 was considered statistically significant. Asterisks (∗) in tables and figures indicate statistical differences of preveraison (PRE) and postveraison (POST) leaf removal treatments with respect to CANOPY controls.

Chapter 3
Experimental monitoring – phenology and vineyard measurements

3.1 Introduction

The overall aim of our study is to understand the effect that basal leaf removal has on amino acid accumulation in Sauvignon blanc grapes and to investigate the expression of genes related to nitrogen assimilation and proline/arginine metabolism. In order to do this, leaf removal experiments were conducted on Sauvignon blanc vines in the Lincoln University vineyard during two subsequent growing seasons in 2013 and 2014. Three different treatment conditions were utilised, a CANOPY control treatment (canopy leaves maintained), preveraison leaf removal (PRE) and postveraison leaf removal (POST) treatments. In the 2018 growing season, an additional leaf removal experiment was set up in the Lincoln University vineyard in order to provide fresh grape material for OAT enzyme assays (see Chapter 6). The vineyard experiment in 2018 used just two treatment conditions, a control (CANOPY) treatment and a preveraison leaf removal (PRE) treatment. A comprehensive explanation of all experiments and treatments, including timing of application and photographs are described in Chapter 2.1.1.

The results presented in this chapter involve tracking the progress of the above experiments and addressing the effects of basal leaf removal treatments on berry phenology and grape bunch microclimate. Monitoring berry phenology components provides a way of following berry development and maturity, while also defining the key developmental switch of veraison to standardise treatments and optimise samplings and results. Berry phenology measurements were also used to investigate any potential effects of leaf removal treatments on berry development. Basal leaf removal alters the source-sink balance and it was of particular interest to demonstrate if a change in the local leaf canopy influences TSS accumulation in the grape bunches (as an indication of berry maturity). Given that basal leaf removal also changes the level of direct sunlight exposure on the grape bunches, the physical consequences of leaf removal on the exposed grapes was observed and approximated temperature parameters were also monitored.

3.2 The grapevine as a perennial plant and leaf removal experimental design

To avoid any potential issues in our experiments of disrupting balances in carbohydrate accumulation, partitioning and storage, the vine blocks used for our leaf removal experiments in the first season, were not used for experiments in the subsequent season. This removed the variable of any differences seen in measured metabolite (carbohydrate, amino acids) concentrations or in related gene expression, being due to the impact of canopy defoliation in the previous season.

As the grapevine is a perennial woody plant, during the winter (dormant) season, the vine is wholly dependent on stored reserves of carbohydrate as no photosynthesis is taking place, and won't be until new shoots and leaves emerge and mature in the next growing season (Mullins et al. 1992). Stored reserves of carbohydrates are also required to drive this early shoot and leaf growth until the new leaves are photosynthetically active and able to export assimilates for grapevine leaf growth and fruit development in the new growing season. Factors that affect the ability of the vine to produce and/or accumulate reserves of carbohydrates have been shown to influence how the vine performs in the following season (Edson et al. 1993; Eltom et al. 2013). In particular, vineyard leaf removal experiments have demonstrated that defoliation results in less carbohydrate reserves in the vines and this has a follow-on effects on vine development in the subsequent growing season (Bennett et al. 2005; Holzapfel et al. 2006; Petrie et al. 2000). Such previous experiments reflect the importance of maintaining carbohydrate reserves and taking into consideration the perennial nature of the grapevine when designing experiments on vines in the field.

3.3 The effect of leaf removal treatments on berry phenology

Measurements were taken throughout the time course of each experiment in the 2013 and 2014 seasons to monitor grape development parameters. Results are reported in both; day of the year (DOY), and days postveraison (DPV) time points. The DOY reporting gives an indication of how berry development and maturity proceeded with respect to previous seasons. Berry phenology measurements were also used to indicate timing for the application of the experimental leaf removal treatments. The date of veraison and accompanying berry phenology dictated the application of postveraison leaf removal (POST) treatment timings. The DPV reporting allowed a standardisation of berry development measurements between seasons, with respect to the developmental stage of veraison. DPV timings also was used to standardise samplings between seasons and subsequently, amino acid measurements, gene expression and enzyme assay results in the following chapters.

3.3.1 Total soluble solids (°Brix)

The accumulation of total soluble solids (TSS), measured as degrees Brix (°Brix), were determined throughout each experiment (season) to provide an indication for berry development and maturity. And as mentioned above, monitoring °Brix levels identified the key developmental switch of veraison, which aided the timings of the leaf removal treatments to the vines. A total soluble solids concentration of 8°Brix was therefore used as a measure of veraison to standardise treatments and results between seasons. A measurement of 8°Brix has previously been used to define the midpoint of veraison and has shown to correspond to maturation onset in Sauvignon blanc (Gregan & Jordan 2016; Parker et al. 2014).

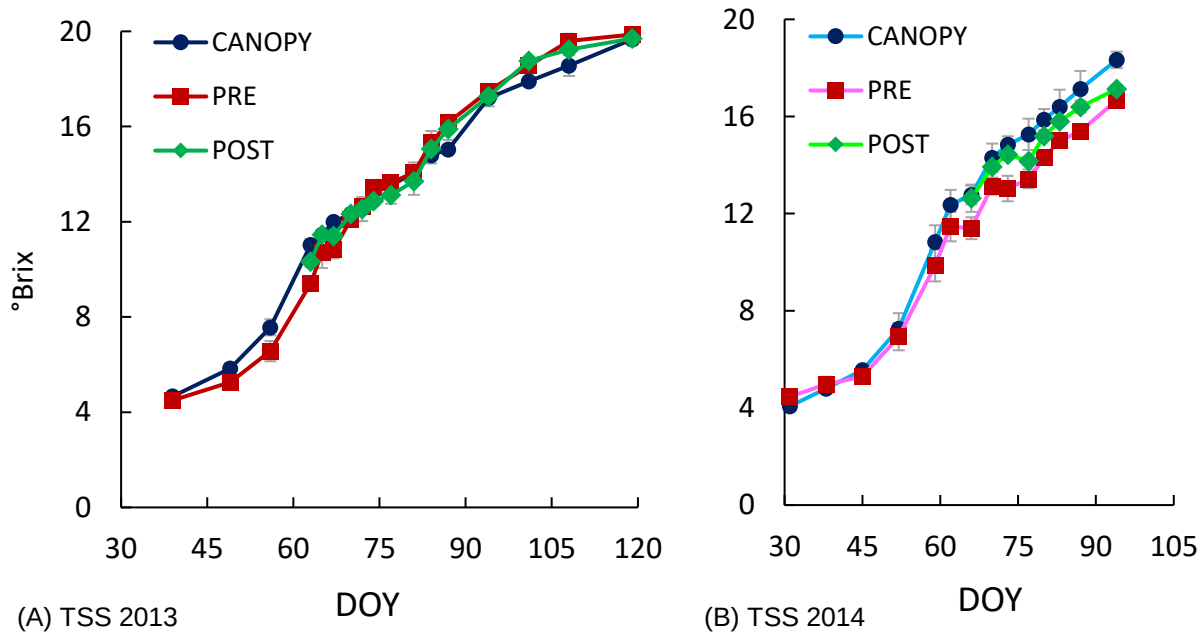


Figure 3.1 Effect of basal leaf removal on TSS accumulation.

TSS accumulation (°Brix) in Sauvignon blanc grapes during berry development comparing CANOPY controls and (PRE and POST) leaf removal treatments. (A) 2013 season and (B) 2014 season. Sampling times are represented as day of the year (DOY). Each data point is the mean ± SEM (n = 3).

In the 2013 season, the PRE treatment initially showed a small decrease in TSS accumulation following application of the leaf removal compared to the CANOPY control treatment, delaying veraison (8°Brix) by 3 days. (Figure 3.1 and Table 3.1). By DOY 70 (13 DPV), PRE TSS levels had recovered to be the same as the CANOPY control levels. Accumulation of TSS in the PRE treatment then continued at the same rate as the CANOPY control and at harvest (DOY 119), was equivalent to the CANOPY treatment. Postveraison leaf removal (POST) had no effect on the berries ability to accumulate TSS through berry development, Brix levels essentially tracking the CANOPY treatment from DOY 65 through to harvest (Figure 3.1).

Conversely to 2013, at harvest in 2014, the PRE and POST treatments had less accumulated TSS than the CANOPY control. This was a postveraison effect and by harvest at DOY 94, the CANOPY control was 1.6°Brix and 1.2°Brix higher than the PRE and POST leaf removal treatments, respectively. Initially following leaf removal early in development, there was no difference between the CANOPY control and PRE treatments, both reaching veraison (8°Brix) at DOY 54. From veraison onwards, the PRE berries, accumulated TSS at a slower rate through the postveraison ripening phase to harvest (DOY 94). It is unknown if the berries had been allowed to ripen for longer if these differences between the treatments would have persisted.

Table 3.1 Monitoring of developmental stages in vineyard experiments.

Day of the year (DOY) and days postveraison (DPV) at experimental/berry development stages comparing CANOPY controls and (PRE and POST) leaf removal treatments. Experiment/development stages are treatment setup, verasison (V), 16°Brix, 18°Brix and final sampling. Also shown is the TSS (°Brix) at the final sampling comparing seasons and treatments.

		Day of the year (DOY)						°Brix
Year	Treatment	CANOPY/PRE setup	V	POST setup	16°Bx	18°Bx	Final sampling	Final sampling
2013	CANOPY	39	57	-	90	102	119	19.7
	PRE	39	60	-	87	97	119	19.9
	POST	-	-	63	88	97	119	19.7
2014	CANOPY	31	54	-	80	92	94	18.3
	PRE	31	54	-	90	-	94	16.7
	POST	-	-	66	84	-	94	17.1
2018	CANOPY	17	44	-	73	82	101	21.0
	PRE	17	46	-	76	84	101	21.2
		Days postveraison (DPV)						°Brix
Year	Treatment	CANOPY/PRE setup	V	POST setup	16°Bx	18°Bx	Final sampling	Final sampling
2013	CANOPY	-18	0	-	33	45	62	19.7
	PRE	-18	3	-	30	40	62	19.9
	POST	-	-	6	31	40	62	19.7
2014	CANOPY	-23	0	-	26	38	40	18.3
	PRE	-23	0	-	36	-	40	16.7
	POST	-	-	12	30	-	40	17.1
2018	CANOPY	-27	0	-	29	38	57	21.0
	PRE	-27	2	-	32	40	57	21.2

Values are means (n = 3).

The experiment in 2014 was harvested earlier and at slightly less CANOPY control Brix levels than in 2013 (see Table 3.1). This was due to some unfavourable weather late in the growing season in 2014 (see Figure 3.5) which included some unseasonably cold weather, heavy rain, frosts and increased disease pressure. To minimise the impact on our experiment and reduce the risk of these climatic conditions adversely affecting our results, the experiment was finished at DOY 94 (40 DPV) with the CANOPY control at an average of 18.3°brix. The experiment in 2013 ran for a further 22 days (to 62 DPV) with a 19.7°Brix in CANOPY controls at harvest.

3.3.2 Berry weight

It is important to take other measures of berry phenology, such as berry weight into account. Aside from the accumulation of TSS, differences in berry weight could also indicate a significant effect of our treatments on berry development. Changes to berry weight was followed through development in both 2013 and 2014 and is shown in Figure 3.2.

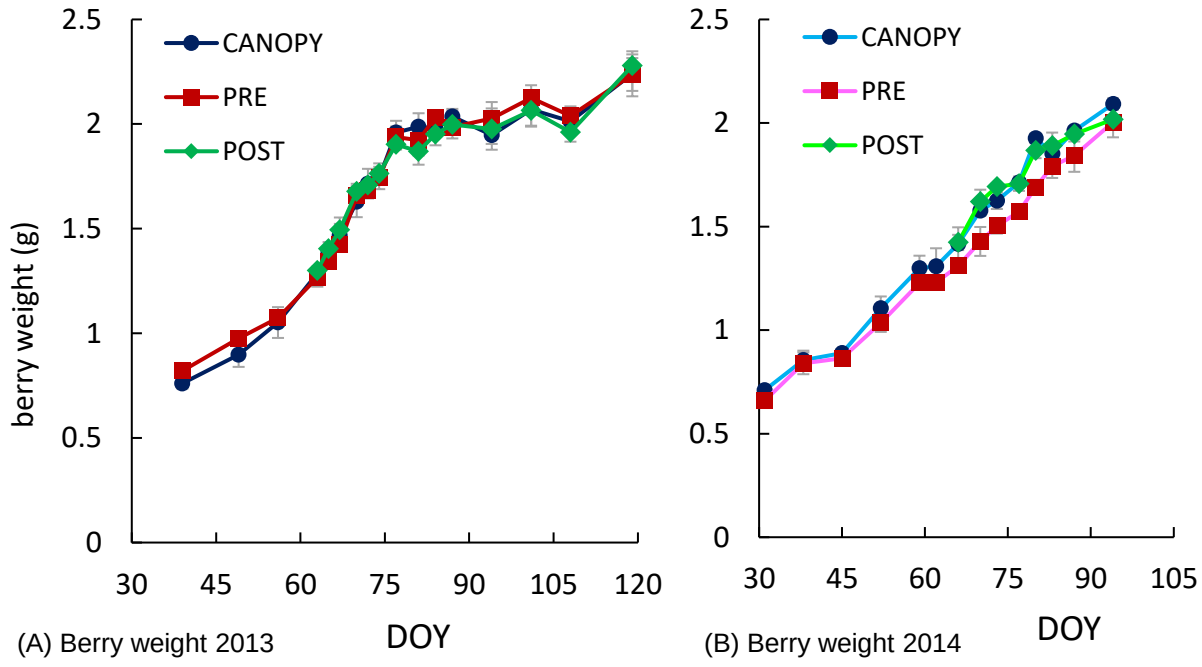


Figure 3.2 Effect of basal leaf removal on berry weight.

Berry weights in Sauvignon blanc grapes during berry development comparing CANOPY controls and (PRE and POST) leaf removal treatments. (A) 2013 season and (B) 2014 season. Sampling times are represented as day of the year (DOY). Each data point is the mean ± SEM (n = 3).

In 2013, berry weight in the CANOPY control treatment increased sharply through veraison, more than doubling in weight from 0.9 g at DOY 49 to 2.1 g at DOY 77. From this point, berry weight plateaus, increasing at a much slower rate to be 2.2 g by DOY 119 (harvest). Berry weight remained unaffected by either the PRE or POST leaf removal treatment.

The rate of increase in berry weight in 2014 was generally slower when compared to 2013. The steep increase through veraison was not observed, and on a whole, the rate of increase was more consistent through development. Although, at completion of the experiment in 2014 (DOY 94) berry weight in the control was 2.1 g and equivalent to 2013 at the same time point and developmental stage. Similarly to the accumulation of TSS, preveraison leaf removal had a small effect on berry weight in 2014 . And again like TSS accumulation, this influence was postveraison, the decrease in berry weight of the PRE treatment occurring only from veraison onwards. However, by the final sampling on DOY 94, no differences between treatments were observed. Postveraison leaf removal did not affect berry weight.

3.4 Physical consequences of leaf removal

The most obvious physical consequence of exposure of the fruit following the leaf removal treatments, was the appearance of increased pigmentation in localised spots on the skins of the berries. The increase in pigmentation on the berry skins occured after veraison, as no observable differences were noted preveraison (Figure 3.3).

This physical response to light exposure postveraison was also observed on grapes of bunches that were naturally outside of the leaf canopy in the control treatments. Bunches (or even part bunches) protected from direct light exposure and within the shade of the leaf canopy showed little pigmentation. Often the pigmentation with light exposure, and lack of with shading, was seen on berries from the same bunch. The observations of pigmentation appearing postveraison with light exposure, is consistent with previous results performed in Sauvignon blanc grapes (Gregan et al. 2012; Liu et al. 2015).

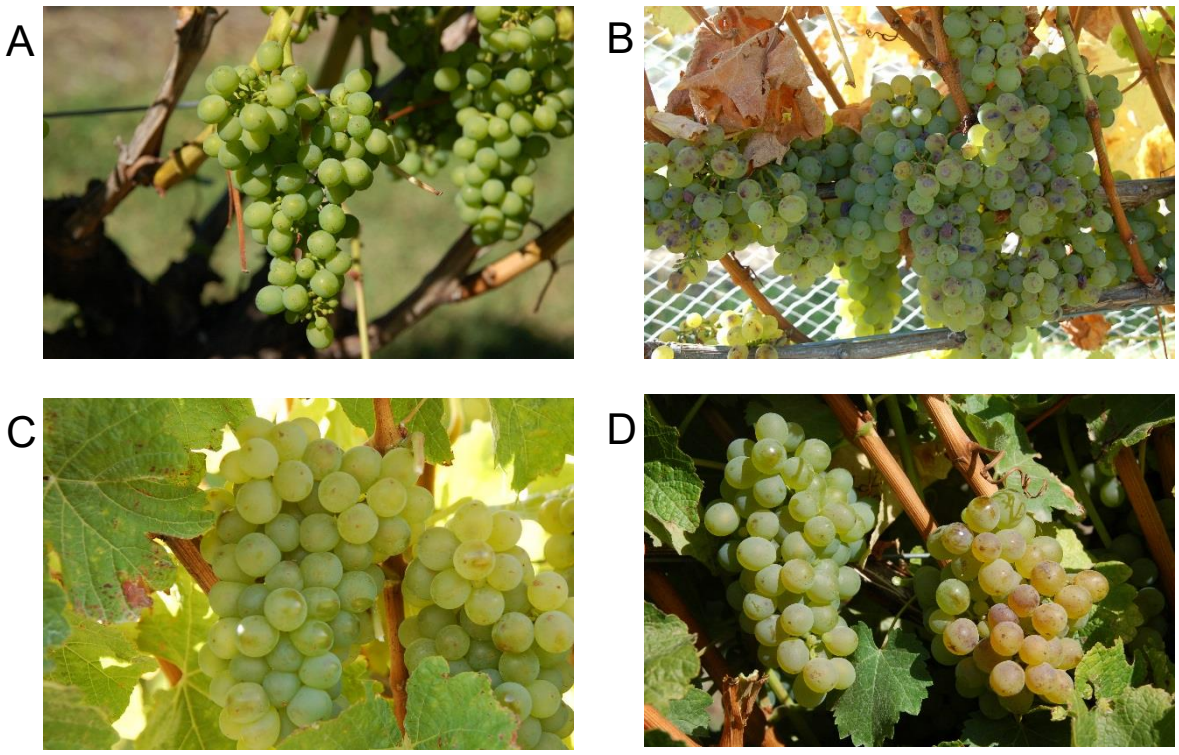


Figure 3.3 Effect of light exposure on berry pigmentation in Sauvignon blanc grapes.

(A) Preveraison grape bunches exposed to sunlight by leaf removal showing no pigmentation. (B) Postveraison grape bunches exposed to sunlight, exhibiting UV damage in the form of “sunburn” and localised pigment spots on the berry skins. (C) Postveraison grape bunches protected from sun exposure by a maintained leaf canopy (control treatment) showing minimal UV damage. (D) An example of postveraison grape bunches in the control treatment demonstrating the effect of differential light exposure. The bunch on the left in the shade of the leaf canopy and has little pigmentation. The bunch on the right is “naturally” sitting outside of the leaf canopy and shows UV damage due to sunlight exposure.

3.5 2018 vineyard experiment

In 2018, a third vineyard experiment was set up in the Lincoln University vineyard during that growing season with two treatment conditions being used, a CANOPY control treatment and a preveraison leaf removal (PRE) treatment. The treatments in 2018 were applied to the vines in the same manner as the 2013 and 2014 vineyard experiments, with CANOPY control treatments maintaining the grapevine canopy leaves around the fruit bunches, and the preveraison leaf removal treatment removing the basal shoot leaves. The main purpose of the 2018 vineyard experiment was to provide fresh grape berry material for ornithine aminotransferase (OAT) enzyme assays. Frozen berries were shown to be not enzymatically active and so berry material used was required to be freshly sampled and not previously frozen (see Chapter 6).

3.5.1 TSS (°Brix) and berry weight in the 2018 season

As in the previous seasons, the accumulation of TSS (°Brix) and berry weight were followed through the 2018 growing season. This again provided an indication for berry development and maturity while aiding the timing of samplings to provide grape material for OAT enzyme assays. The measure of veraison was again used to standardise treatments between seasons and provided a means to compare sampling time points to equivalent developmental samplings in 2013 and 2014.

In 2018, preveraison leaf removal had no effect on the berries ability to accumulate TSS through berry development. Berry weight in 2018 was also unaffected by the preveraison leaf removal treatment (Figure 3.4). However, berry weights in the CANOPY control and PRE treatments in 2018 were significantly increased at all stages of development when compared to the equivalent developmental time points in the 2013 and 2014 seasons. For example, berries in the 2018 sampling were 48% and 35% bigger at veraison (DOY 44, 0 DPV) than in 2013 (DOY 57, 0 DPV) and 2014 (DOY 54, 0 DPV) respectively.

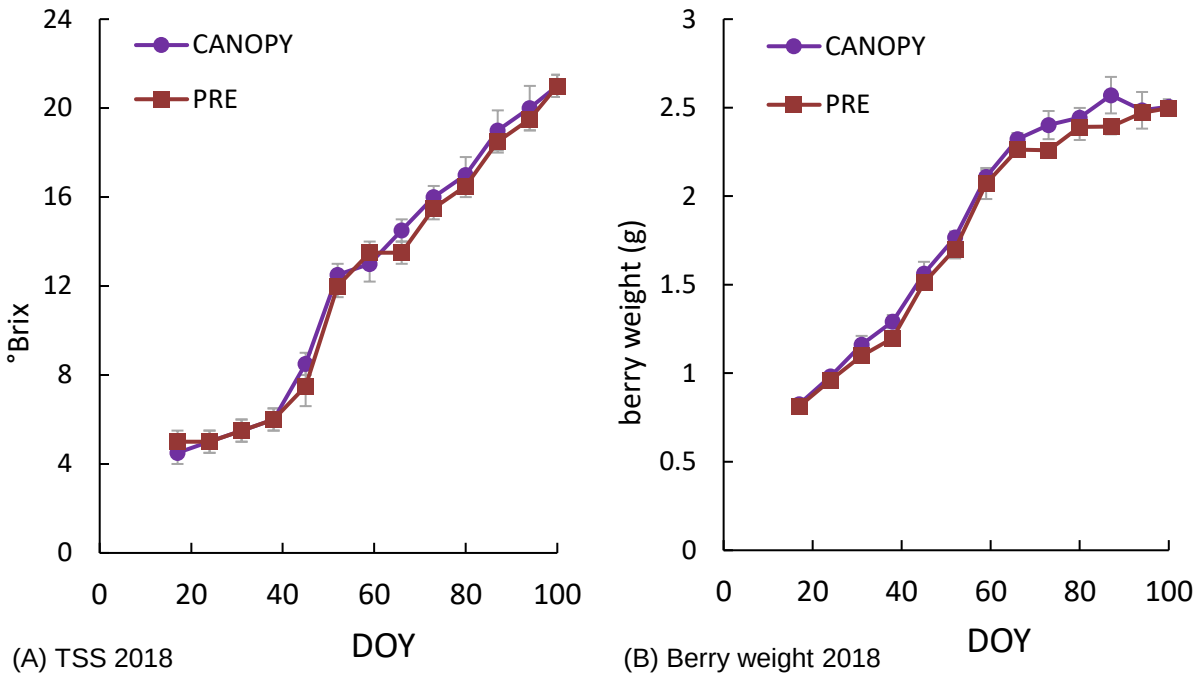


Figure 3.4 Effect of basal leaf removal on TSS accumulation and berry weight.

TSS accumulation (°Brix) and berry weight in Sauvignon blanc grapes during berry development, comparing CANOPY controls and (PRE) leaf removal treatments. (A) TSS 2018 season and (B) Berry weight 2018 season. Sampling times are represented as day of the year (DOY). Each data point is the mean ± SEM (n = 3).

Aside from significant seasonal differences in berry weight, berries in 2018 also accumulated TSS earlier compared to the 2013 and 2014 seasons. The developmental stages of veraison, 16°Brix and 18°Brix were reached earlier in the growing season at DOY 44, DOY 73 and DOY 82 respectively. However, when sampling times were converted to DPV, the transition from veraison to 16°Brix and 18°Brix showed no significant differences compared to the 2013 and 2014 seasons (Table 3.1).

3.6 Vineyard climate data

3.6.1 Bunch microclimate

CANOPY control treatments were essentially untouched vines with a complete maintenance of all basal canopy leaves. The physiological result of the PRE and POST treatments was to alter the source/sink balance and fully expose the fruit at different stages of berry development. The fruiting zone of the CANOPY control treatment which was under the shade of the maintained leaf canopy, received minimal photosynthetic photon flux and UV index values. This was is in stark comparison to the PRE and POST treatments, whereby exposed fruit was often in full sun with values equivalent to the normal seasonal values (see Gregan et al. (2012), data not shown).

Aside from the obvious difference between CANOPY controls and leaf removed (PRE and POST) vines in the modification of the leaf source to berry sink ratio and bunch light exposure, other aspects of bunch microclimate of the grape bunches were altered. Leaf removal in the PRE and POST treatments allowed wind and airflow to penetrate the vines and circulate around the grape bunches to a greater extent compare to the full leaf canopy. Although not quantified, other factors such as bunch temperature and humidity will have been impacted.

NB. In order to measure approximate differences in bunch microclimate temperatures between the CANOPY control and leaf removal treatments, temperature microloggers were used in the 2018 vineyard experiment. The results are presented below in Chapter 3.6.3, but are also applicable to the 2013 and 2014 vineyard experiments.

3.6.2 Accumulated growing degree days (GDD)

In the absence of extreme conditions such as drought or disease, plant growth is strongly influenced by the ambient air temperature. Accumulated growing degree days (GDD) is a temperature based indicator and provides a measure of heat accumulation through a growing season. GDDs are useful for comparing the progress of a current growing season to the long-term average and are sometimes used as a model for estimating crop development stages and maturity dates. They are also useful for comparing between seasons and can, in the example of grapevine, be indicative of differences in berry phenology and berry composition (Gregan & Jordan 2016).

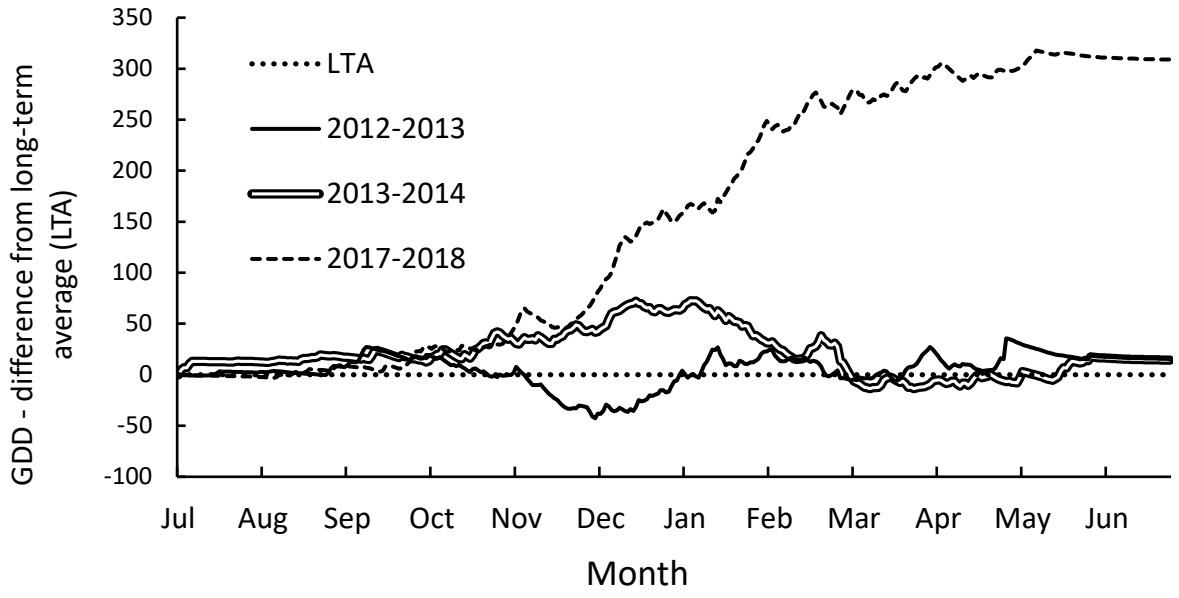


Figure 3.5 Accumulated growing degree days (GDD) in the Lincoln University vineyard.

Accumulated GDDs showing differences to the long-term average (LTA) from July through to the end of June during the 2012/2013, 2013/2014 and 2017/2018 seasons. The LTA (baseline 0) is indicated by the horizontal dotted line.

By the end of the growing seasons in both 2013 and 2014 (March-May), the accumulated GDD in both seasons were equivalent to the LTA (Figure 3.5). The main difference between 2013 and 2014 was the period between November to February where the GDD in 2013 tracked below the LTA and the GDD in 2014 tracked above the LTA. By February, the difference between the two seasons had closed and from here, both tracked each other and the LTA. In 2014, from January, the GDD decreased sharply to be below the LTA by March/April. As described above, some adverse weather conditions during this growing season and later in berry development contributed to this observation. This meant that the experiment in 2014 was finished earlier (as measured in DPV) compared to 2013, to minimise the impact

on our experiment and reduce the risk of these "colder" conditions adversely affecting the fruit and hence, our results. However, in 2014, the time it took for the fruit in the CANOPY control treatments to reach 16 and 18°Brix was accelerated compared to 2013, even with less GDD. This was reflected in the transition from veraison to 18°Brix occurring at 38 DPV with a DD of 896, compared to 45 DPV and a GDD of 948 in 2013 (Table 3.2).

The GDD in 2018 moved above the LTA in November (before grapevine flowering) and continued accumulating to track significantly higher than the LTA (and the 2013 and 2014 seasons) right through the growing season (Figure 3.5 and Table 3.2).

Table 3.2 Accumulation of GDDs in vineyard experiments.

Accumulated growing degree days (GDD) at experimental/berry development stages comparing CANOPY controls and (PRE and POST) leaf removal treatments. Experiment/development stages are treatment setup, veraison (V), 16°Brix, 18°Brix and final sampling. Also shown is the TSS (°Brix) at the final sampling comparing seasons and treatments.

		Accumulated growing degree days (GDD)						°Brix
Year	Treatment	CANOPY/PRE setup	V	POST setup	16°Bx	18°Bx	Final sampling	Final sampling
2013	CANOPY	630	730	-	917	948	986	19.7
	PRE	630	753	-	895	938	986	19.9
	POST	-	-	768	901	938	986	19.7
2014	CANOPY	596	745	-	853	896	906	18.3
	PRE	596	745	-	886	-	906	16.7
	POST	-	-	781	866	-	906	17.1
2018	CANOPY	641	899	-	1095	1149	1233	21.0
	PRE	641	915	-	1115	1153	1233	21.2

Values are means (n = 3).

3.6.3 Bunch temperature microclimate

Leaf removal treatments have the effect of exposing the grape bunches to direct sunlight thereby increasing the amount of radiant heat reaching the fruit. Bunches shaded by the leaf canopy in the CANOPY control treatments are not exposed to the same amount of radiant sunlight energy and are comparatively protected. In order to measure approximate differences in average temperatures between the CANOPY control and leaf removal treatments, temperature microloggers were used in the 2018 vineyard experiment. Microloggers were placed hanging from canes alongside the grape bunches and either naturally shaded by the leaf canopy in the CANOPY control treatments, or fully exposed (and sometimes in the direct sunlight) alongside exposed bunches in the PRE treatment (see Chapter 2.3.3).

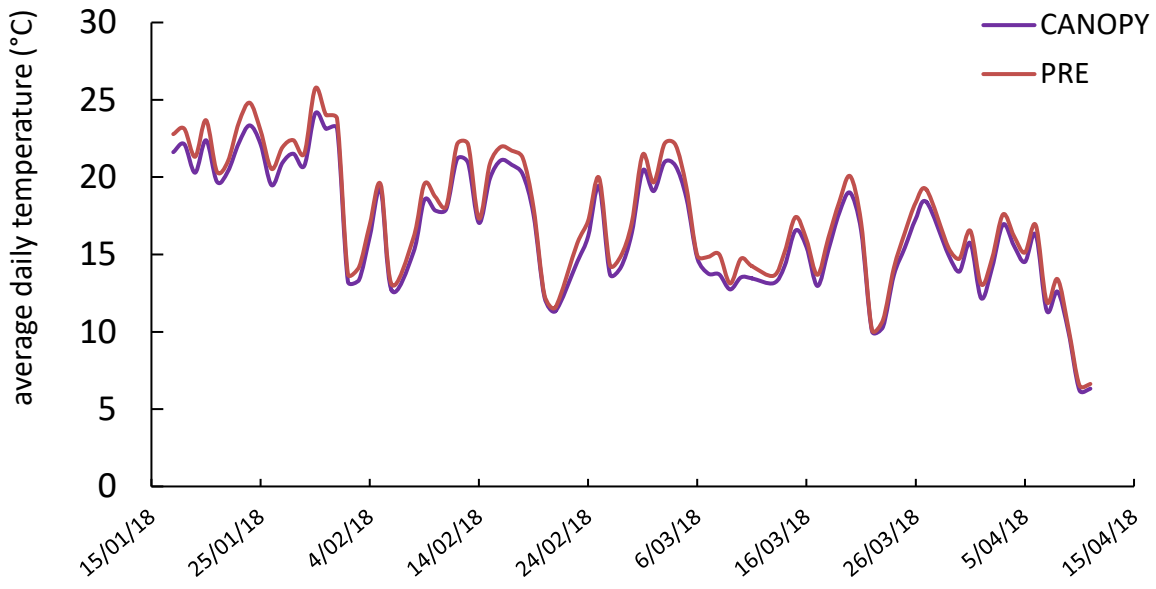


Figure 3.6 Average daily temperatures during the 2018 vineyard experiment.

Average daily temperatures comparing CANOPY controls and preveraison leaf removal (PRE) treatments. Measurements were made using temperature microloggers placed alongside grape bunches either naturally shaded by the leaf canopy in the CANOPY control treatments, or fully exposed alongside exposed bunches in the PRE treatment. Each data point is the mean of the pooled replicates (n = 6).

Overall, the average daily temperatures measured by the microloggers was increased (Figure 3.6) in the PRE treatment. The average daily temperature during the sampling period of the experiment (DOY 17-101) was increased by an average of: 1.0°C during January; 0.7°C in February; 0.8°C in March; and 0.6°C during April, as measured by the microloggers placed beside the exposed grape bunches in the PRE treatment, compared to shaded controls. Figure 3.7 shows a subset of data across two indicative 10 day

periods from 18 January - 28 January 2018 and 1 March - 11 March 2018, respectively. It clearly shows that the differences in average daily temperature was due to an increase in maximum temperatures due to direct sunlight exposure during the day. There was no difference in measured temperatures during the early mornings, late evening or overnight. To validate this observation, minimal differences were noted between the treatments on “cooler” and cloudy days.

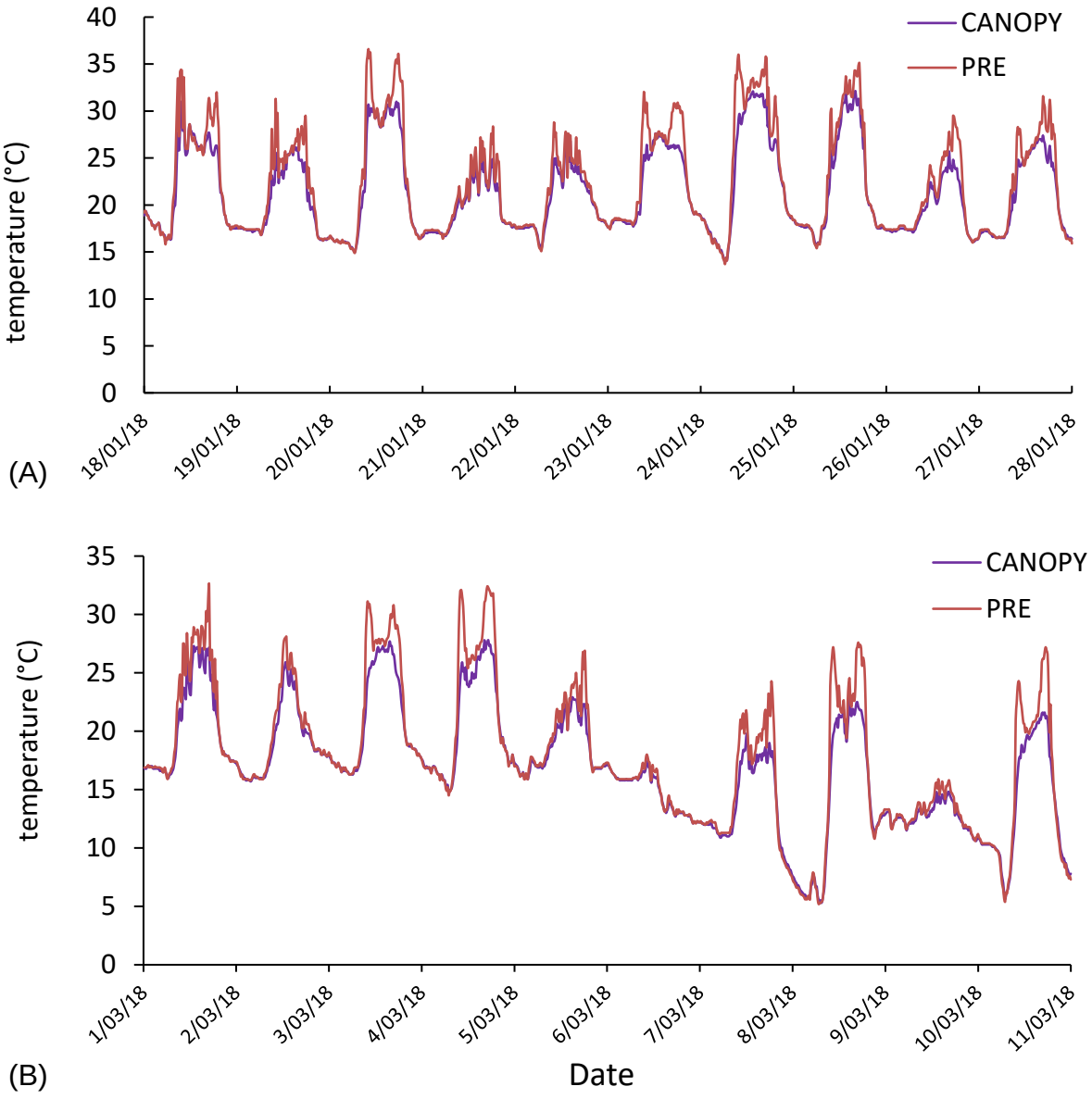


Figure 3.7 Daily temperature variations during the 2018 vineyard experiment.

Daily temperature variations comparing CANOPY controls and preveraison leaf removal (PRE) treatments during two 10 day subsets of the 2018 vineyard experiment; (A) 18 January - 28 January 2018, and (B) 1 March - 11 March 2018. Measurements were made every 15 min using temperature microloggers placed alongside grape bunches either naturally shaded by the leaf canopy in the CANOPY control treatments, or fully exposed alongside exposed bunches in the PRE treatment. Each data point is the mean of the pooled replicates (n = 6).

3.7 Conclusions

The results presented above involved monitoring berry phenology measurements. This provided an indication of how berry development and maturity might potentially be influenced by the basal leaf removal treatments. Generally our results indicate that basal leaf removal has a minimal impact on TSS accumulation in developing Sauvignon blanc grape berries. Nevertheless, the result in 2014 indicates that there is the potential for basal leaf removal to have small effects on TSS accumulation. The preveraison and postveraison leaf removal treatments had a minimal effect on the berry phenology measurement of berry weight.

The basal leaf removal treatments also considerably alters the local bunch microclimate. An increase in light exposure on the grape bunches, demonstrated a change in the physical appearance of the berries from the leaf removal treatments. Additionally, temperature microloggers placed alongside exposed grape bunches (from leaf removal treatments) showed an increase in average temperatures compared to microloggers in the full shade of the CANOPY controls, due to the direct sunlight exposure on the microloggers in the middle part of the day.

Chapter 4

Amino acid accumulation in Sauvignon blanc grapes – the quantitative and qualitative responses to basal leaf removal

4.1 Introduction

As elaborated in Chapter 1, leaf removal is a common viticultural management tool used in commercial vineyards. We are using this well-established viticultural intervention as an experimental technique to investigate the effect on amino acid biochemistry in Sauvignon blanc grapes. As well as altering the local bunch microclimate (presented in Chapter 3), basal leaf removal removes a significant source of assimilates proximal to the grape bunches, which in turn has the effect of modifying the source-sink relationship. The aim of the experiments presented in this chapter are to investigate this balance by removing basal leaves at preveraison and postveraison stages of development. Our hypotheses are that basal leaf removal on the lower portion of the shoot and around the fruiting zone of the vine will modify the source (leaf) - sink (grape bunches) relationship and affect amino acid accumulation in the fruit and potentially, alter qualitative aspects of accumulation.

The monitoring of berry phenology described in Chapter 3, demonstrated that in our experiments basal leaf removal had no effect on TSS accumulation and berry weight in both the 2013 and 2018 seasons, and only a minimal effect in 2014. This important finding shows that basal leaf removal did not significantly alter berry phenology and hence, berry development and maturity. It informs the next phase of this research, as at this stage we can extrapolate that any potential differences observed in amino acid accumulation is likely to arise from the effect of our experimental leaf removal treatments and not from a delay or retardation of berry development. It also suggests that, at least for TSS accumulation, that other parts of the canopy (i.e. younger apical leaves and laterals) can “compensate” for the loss of a local source of leaves and assimilates.

This results of this chapter describe the measurements following amino acid accumulation throughout berry development. Amino acid concentrations were determined for Sauvignon blanc grape berry samples collected throughout the growing seasons of 2013 and 2014 at multiple time points. CANOPY control treatments (a maintenance of canopy leaves) were compared to preveraison (PRE) and postveraison (POST) leaf removal treatments to determine the effect of leaf removal on total amino acid concentrations in Sauvignon blanc grapes; and also the effect on the individual amino acid profiles of accumulation through development. Also investigated was the influence that leaf removal has on the qualitative nature of individual amino acid accumulation, measured as the proportion (percentage) of total amino acid concentrations. All results presented in this chapter are reported as DPV, as to

standardise sampling timings and allow comparisons of results between the seasons with respect to the developmental stage of veraison.

4.2 The effect of basal leaf removal on total amino acid concentrations

4.2.1 Total amino acid accumulation in the 2013 season

In 2013, amino acid concentrations were determined at 17 time points through development, from application of the PRE treatment at -18 DPV to harvest at 62 DPV (Figure 4.1 and Appendix 3). Overall, from -18 DPV to 62 DPV, total amino acid concentrations in the CANOPY control increased 59%. Whereas in the same period in the PRE treatment and from 6 DPV to 62 DPV in the POST treatment, total amino acid concentrations only increased 37% and 25% respectively (Table 4.1). In the CANOPY control treatment, initially total amino acid concentration falls 17% from 16429 µmol/L at -18 DPV to 13618 µmol/L at veraison (-1 DPV). However, from veraison onwards, total amino acid concentration increases significantly through berry ripening to 25009 µmol/L by 27 DPV (*P*-value <.001). There is another period of decline from 27 DPV to 44 DPV where total amino acids decrease to 21490 µmol/L, before going through another period of accumulation to be 26109 µmol/L at 62 DPV.

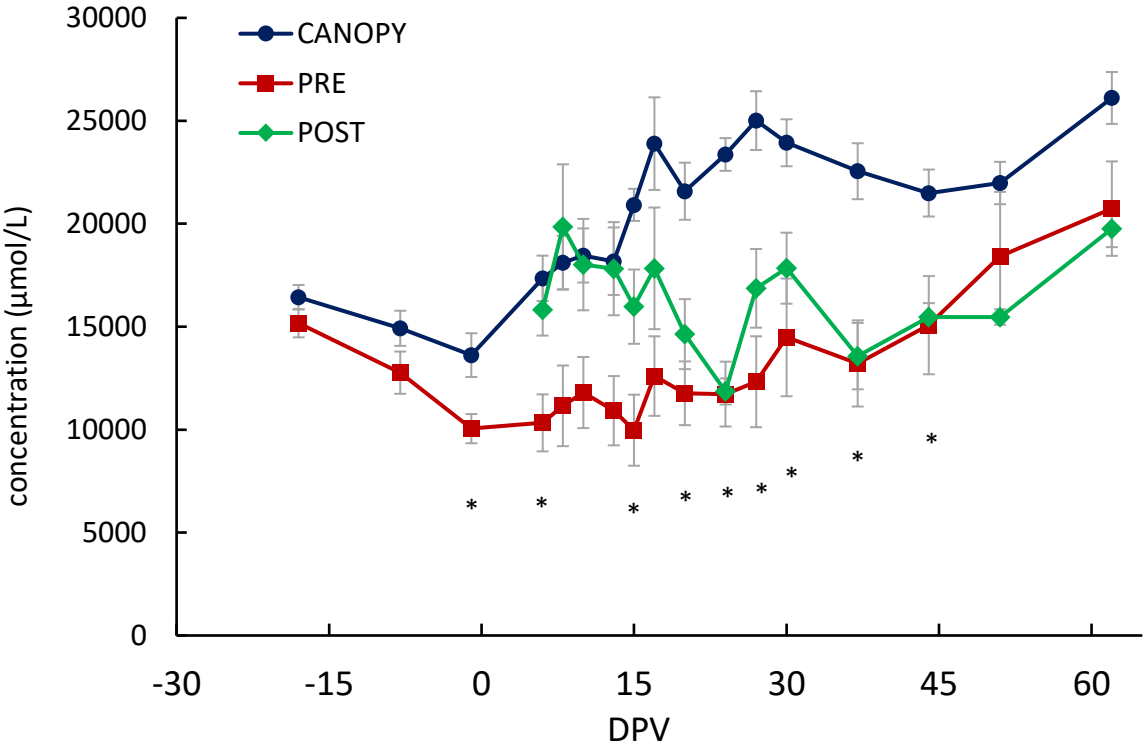


Figure 4.1 The effect of basal leaf removal on total amino acid accumulation (2013 season).

Total amino acid concentrations in Sauvignon blanc grapes during berry development, comparing CANOPY controls and (PRE and POST) leaf removal treatments. Sampling times are represented with respect to veraison (DPV, days postveraison). Each data point is the mean ± SEM (n = 3). Asterisks (*) indicate statistical differences of treatments using ANOVA (*lsd* at 5% level, see Appendix 3).

Table 4.1 Changes in total amino acid accumulation (2013 season).

Percentage increases/decreases in total amino acid concentrations in Sauvignon blanc grapes during berry development, comparing CANOPY controls and (PRE and POST) leaf removal treatments. Total amino acid concentrations comparing (PRE and POST) leaf removal treatments as a percentage of the CANOPY controls.

2013 season	Percentage increase/decrease (%)*			Percent of CANOPY control (%)**		
Days postveraison (DPV)	Treatment			Treatment		
	CANOPY control	**PRE**	**POST**	**CANOPY control**	**PRE**	**POST**
-18	0	0	-	100	92	-
-8	-9	-16	-	100	86	-
-1	-17	-34	-	100	74	-
6	6	-32	0	100	60	91
8	10	-26	25	100	62	110
10	12	-22	14	100	64	98
13	11	-28	13	100	60	98
15	27	-34	1	100	48	76
17	45	-17	13	100	53	75
20	31	-22	-8	100	55	68
24	42	-23	-25	100	50	51
27	52	-19	7	100	49	67
30	46	-5	13	100	61	75
37	37	-13	-14	100	59	60
44	31	-1	-2	100	70	72
51	34	21	-2	100	84	70
62	59	37	25	100	79	76

*Values are means (n=3). Percentages were calculated through development with respect to the amino acid concentrations on day of application of the treatment; -18 DPV for CANOPY control and PRE, and 6 DPV for POST. **Values are means (n=3). Percentages were calculated at each time point through development, comparing PRE and POST amino acid concentrations with the CANOPY control.

On the whole, basal leaf removal significantly reduces total amino acid accumulation throughout development (Figure 4.1 and Table 4.1). In the PRE treatment berries following leaf removal, total amino acid concentration initially decreases from -18 DPV through to veraison (-1 DPV). This was a similar trend as observed in the CANOPY control, however, the decrease was greater in the PRE treatment, total amino acids decreasing 34% to be 10047 µmol/L at veraison. Total amino acids accumulate then at a much slower rate in the PRE treatment through the ripening phase, compared to the CANOPY control. It is not until sampling 15 at 44 DPV that total amino acid concentrations in the PRE treatment berries recover to be equivalent to their -18 DPV levels. However, the PRE treatment does go through a period of more significant accumulation later in development from 37 DPV to 62 DPV DPV (*P*-value <.001), increasing 57% from 37 DPV to be 20734 µm by harvest (62 DPV). The biggest differences between

CANOPY control and PRE treatment accumulation of amino acids occurs between 6 DPV and 37 DPV. During this mid-ripening stage of development, total amino acid concentration in the PRE berries ranged between 48% to 64% of CANOPY control levels. Due to the later surge of accumulation in the PRE treatment from 37 DPV, these proportions did increase and at 62 DPV, total amino acid concentration in the PRE berries was 79% of the CANOPY control (Table 4.1).

From application of the postveraison leaf removal treatment at 6 DPV, overall, the total amino acid concentration in the POST berries decreases to be equivalent with the PRE treatment at 37 DPV (13570 µmol/L) . The POST treatment then followed the PRE treatment with an equivalent accumulation of amino acids from 37 DPV to 62 DPV. Similarly to the PRE treatment, the greatest differences between the control and POST treatment amino acid concentrations occurs during the mid-ripening phase of berry development from 20 DPV to 37 DPV. Total amino acid concentrations are significantly decreased during this stage of berry development in the POST berries being as low as 51% of the control at 24 DPV (Table 4.1).

4.2.2 Total amino acid accumulation in the 2014 season

In 2014, amino acid concentrations were determined at 14 time points through development, from application of the PRE treatment at -23 DPV to harvest at 40 DPV (Figure 4.2). Overall, from -23 DPV to 40 DPV, total amino acid concentrations in the CANOPY control increased 35%. Whereas in the same period in the PRE treatment and from 12 DPV to 40 DPV in the POST treatment, total amino acid concentrations decreased 16% and 8% respectively (Table 4.2). In the CANOPY control treatment in 2014, total amino acids increase through development from 13156 µmol/L at -23 DPV to a peak of 18762 µmol/L at 19 DPV. From 19 DPV, total amino acid concentrations essentially plateau and finish slightly less than the peak, being 17764 µmol/L at 40 DPV.

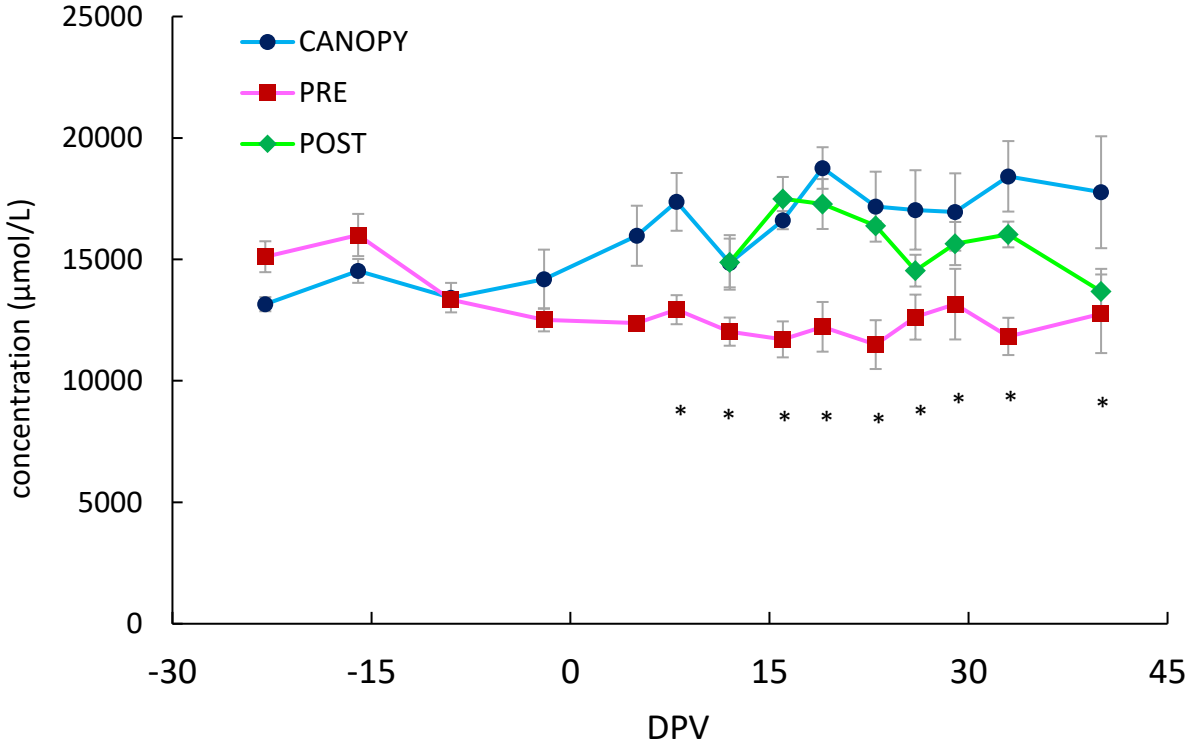


Figure 4.2 The effect of basal leaf removal on total amino acid accumulation (2014 season).

Total amino acid concentrations in Sauvignon blanc grapes during berry development, comparing CANOPY controls and (PRE and POST) leaf removal treatments. Sampling times are represented with respect to veraison (DPV, days postveraison). Each data point is the mean ± SEM (n = 3). Asterisks (*) indicate statistical differences of treatments using ANOVA (*lsd* at 5% level, see Appendix 3).

Similarly to 2013, basal leaf removal significantly reduces total amino acid accumulation throughout development. Initial concentrations of amino acids in the PRE treatment berries are slightly higher than the CANOPY control, peaking at 16003 µmol/L at -16 DPV. Levels in the PRE treatment, then decrease 25% to be 12511 µmol/L at veraison (-2 DPV), less than CANOPY control concentrations of 14185 µmol/L. From -2 DPV through the ripening stage of development, total amino acid concentration in the PRE berries plateau and finishing equivalent to veraison levels, being 12761 µmol/L by 40 DPV. The biggest

differences between the CANOPY control and PRE treatment levels of amino acids in 2014 occurs between 16 DPV and 33 DPV. During this postveraison stage of development, total amino acid concentration in the PRE berries ranged between 64% to 74% of control levels (Table 4.2).

Table 4.2 Changes in total amino acid accumulation (2014 season).

Percentage increases/decreases in total amino acid concentrations in Sauvignon blanc grapes during berry development, comparing CANOPY controls and (PRE and POST) leaf removal treatments. Total amino acid concentrations comparing (PRE and POST) leaf removal treatments as a percentage of the CANOPY controls.

2014 season	Percentage increase/decrease (%)*			Percent of CANOPY control (%)**		
Days postveraison (DPV)	Treatment			Treatment		
	CANOPY control	**PRE**	**POST**	**CANOPY control**	**PRE**	**POST**
-23	0	0	-	100	115	-
-16	10	6	-	100	110	-
-9	2	-12	-	100	99	-
-2	8	-17	-	100	88	-
5	21	-18	-	100	78	-
8	32	-14	-	100	74	-
12	13	-20	0	100	81	100
16	26	-23	18	100	70	105
19	43	-19	16	100	65	92
23	31	-24	10	100	67	95
26	29	-16	-2	100	74	85
29	29	-13	5	100	78	92
33	40	-22	8	100	64	87
40	35	-16	-8	100	72	77

*Values are means (n=3). Percentages were calculated through development with respect to the amino acid concentrations on day of application of the treatment; -23 DPV for CANOPY control and PRE, and 12 DPV for POST. **Values are means (n=3). Percentages were calculated at each time point through development, comparing PRE and POST amino acid concentrations with the CANOPY control.

Following application of the postveraison leaf removal treatment at 12 DPV, overall after a small lag, the total amino acid concentration in the POST berries decreased to be equivalent with the PRE treatment at 40 DPV (13679 µmol/L). The greatest differences between the CANOPY control and POST treatment amino acid concentrations occurs at 40 DPV. Total amino acid concentration is significantly decreased at this final sampling time point in the POST, berries being 77% of the control (Table 4.2).

N.B. As discussed in Chapter 3.3.1, the experiment in 2014 was terminated 22 days earlier than in 2013. It is therefore unknown if amino acid levels would have undergone a later accumulation as observed in 2013.

4.2.3 Comparisons between the 2013 and 2014 seasons

Despite the discrepancy between seasons in the experiment durations, both preveraison and postveraison leaf removal significantly reduced total amino acid accumulation in Sauvignon blanc grape berries. Additionally, even during the longer period of sampling from 37 DPV onwards in the 2013 season, when berries from all treatments continued to accumulate amino acids, the effects of leaf removal reducing amino acid accumulation was maintained.

Comparisons of CANOPY control berries at veraison between the two seasons, show total amino acid concentrations were very similar, 13618 µmol/L (-1 DPV) in 2013 and 14185 (-2 DPV) in 2014. The final sampling time point in 2014 was 40 DPV when total amino acid concentrations in control berries were 17764 µmol/L. At the equivalent time point in 2013, total amino acid concentrations were higher at 22551 µmol/L (37 DPV). However, even with large seasonal differences seen in the CANOPY control berries, this seasonal effect was not observed with respect to the leaf removal treatments, the PRE and POST treatments maintaining similar concentrations between seasons (Figure 4.1 and Figure 4.2). Total amino acid concentrations in the 2013 season at 37 DPV in PRE and POST berries, was 13214 µm and 13570 µmol/L respectively. At the equivalent developmental time point of 40 DPV in the 2014 season, total amino acid concentrations in PRE and POST berries were 12761 µmol/L and 13679 µmol/L respectively.

4.3 Individual amino acids as a proportion of total amino acid concentrations.

Table 4.3 illustrates the proportion (as a percentage) each amino acid contributes to total amino acid concentrations at different stages of development. Shown in the table are the results of the CANOPY control treatment samples, at four indicative sampling time points in 2013; -18 DPV (preveraison), -1 DPV (veraison), 37 DPV (postveraison) and 62 DPV (final sampling). Three equivalent sampling time points from the 2014 season are also shown; -23 DPV (preveraison), -2 DPV (veraison) and 40 DPV (final sampling). The individual percentages clearly show that the α-ketoglutarate family of amino acids dominate the proportions of total amino acids through all stages of development. Glutamine is the major amino acid early in berry development and arginine predominates at later time points.

The context of the individual amino acid proportions are important and are detailed further in the preceding sections of this chapter with results from the separate amino acid metabolic families. Aside from the quantitative differences (as seen with basal leaf removal and total amino acid concentrations), also described is the effect that basal leaf removal treatments have on the qualitative nature of the individual amino acids and their concentrations through development.

Table 4.3 The proportions of individual amino acids through development.

Individual amino acids as a percentage of total amino acid concentrations in Sauvignon blanc grapes during berry development. CANOPY control samples are from four time points in the 2013 season; -18 DPV (preveraison), -1 DPV (veraison), 37 DPV (postveraison) and 62 DPV (final sampling), and from three time points in the 2014 season; -23 DPV (preveraison), -2 DPV (veraison) and 40 DPV (final sampling).

Individual amino acids as a percentage of total amino acid concentrations (%)

Treatment	CANOPY Control - 2013 season				CANOPY Control - 2014		
Developmental time point	Pre-veraison	Veraison	Mid-ripening	Harvest	Pre-veraison	Veraison	Harvest
	-18 DPV	**-1 DPV**	**37 DPV**	**62 DPV**	**-23 DPV**	**-2 DPV**	**40 DPV**
Amino acid							
α-ketoglutarate							
Glutamine	70.5	40.3	9.1	4.5	76.3	46.0	9.5
Glutamate	6.2	7.0	6.0	7.9	5.7	6.2	7.8
Arginine	6.3	18.9	34.1	38.1	2.7	16.1	37.0
Proline	0.4	1.1	7.4	11.0	0.5	0.6	8.4
Aspartate							
Aspartate	2.9	4.1	3.1	1.1	2.7	3.9	3.6
Asparagine	2.7	3.2	0.6	0.3	2.4	2.5	0.5
Threonine	1.4	3.9	10.2	9.3	1.0	3.2	8.1
Isoleucine	0.6	1.0	3.2	3.3	0.4	1.3	1.8
Methionine	0.1	0.3	0.8	0.6	0.0	0.4	0.5
Lysine	0.3	1.2	0.8	0.9	0.1	0.2	0.2
Pyruvate							
Leucine	0.4	1.9	4.6	4.8	0.3	1.7	2.2
Alanine	1.9	5.0	6.1	4.1	1.5	6.1	8.0
Valine	0.4	1.0	3.0	3.2	0.4	1.2	2.1
Aromatic							
Phenylalanine	0.6	1.5	3.7	4.0	0.5	1.3	2.6
Tryptophan	0.6	1.4	1.4	1.3	0.5	1.0	1.0
Tyrosine	0.3	1.1	0.6	0.7	0.2	1.1	0.8
Histidine	1.0	2.1	1.1	1.0	1.0	1.4	1.3
3-phosphoglycerate							
Serine	3.1	4.1	3.9	3.5	3.1	4.6	4.1
Glycine	0.4	1.0	0.4	0.4	0.4	0.9	0.2
Cysteine	0.0	0.0	0.0	0.0	0.0	0.0	0.0

*Values are means (n=3). Percentages were calculated at each time point, by comparing each individual amino acid concentration with the total amino acid concentrations of the CANOPY controls.

4.4 The α-ketoglutarate metabolic family of amino acids

As discussed in detail in Chapter 1; glutamine, glutamate, arginine and proline are metabolically interconnected via their biosynthetic pathways. The formation of glutamine via the GS/GOGAT pathway is the main entry point for nitrogen to be assimilated into organic molecules and introduced into cellular biochemical pathways. The majority of this nitrogen assimilation is taking place in the photosynthetically active leaves, from which it is exported into surrounding sinks such as the developing grape bunches. After its import into grape berries, glutamine combines with α-ketoglutarate to produce two molecules of glutamate, one of which is recycled for further nitrogen (ammonia) assimilation. The second molecule of glutamate can be used for production of other amino acids, and in the case of arginine and proline, as both direct precursors and cofactors in their biosynthetic pathways.

4.4.1 Quantitative and qualitative changes to the α-ketoglutarate amino acids

Glutamine, glutamate, arginine and proline are also the four amino acids that are present at the highest concentrations throughout development, together contributing up to 83% (2013 season) and 85% (2014 season) of total amino acid concentrations in control berry samples preveraison (Figure 4.3). In postveraison berries these proportions decrease, but still maintain a tight range between 55% - 62% of total amino acids in both seasons. At postveraison time points therefore, the proportion of these four α-ketoglutarate family amino acids (as a percentage of total amino acids) is remarkably consistent. Nevertheless, within this, the concentrations (and therefore, proportions) of the individual amino acids (glutamine, glutamate, arginine and proline) each change significantly and contribute differentially through development (*P*-value <.001).

The α-ketoglutarate family shows a quantitative response to basal leaf removal. The individual amino acid concentrations of glutamine, glutamate, arginine and proline that contribute to this family, are all significantly reduced at certain stages of development in Sauvignon blanc grape berries following leaf removal treatments. The results of these individual amino acids are addressed in subsequent sections of this chapter. In addition and to further complicate the quantitative observations, the α-ketoglutarate family are qualitatively regulated by basal leaf removal with their individual proportions (as percentages of total amino acids) being affected by our leaf removal treatments (Figure 4.4). As a collective, the α-ketoglutarate family showed higher proportions with respect to the basal leaf removal treatments in both seasons, compared to the proportions from the CANOPY controls. This observation was noted at postveraison time points. Accordingly, the other amino acids measured (grouped together) contained lower proportions of amino acids postveraison with respect to basal leaf removal treatments, compared to the CANOPY controls (Figure 4.4). The qualitative effects of basal leaf removal on the individual amino acids of the α-ketoglutarate family are shown in Appendix 4.

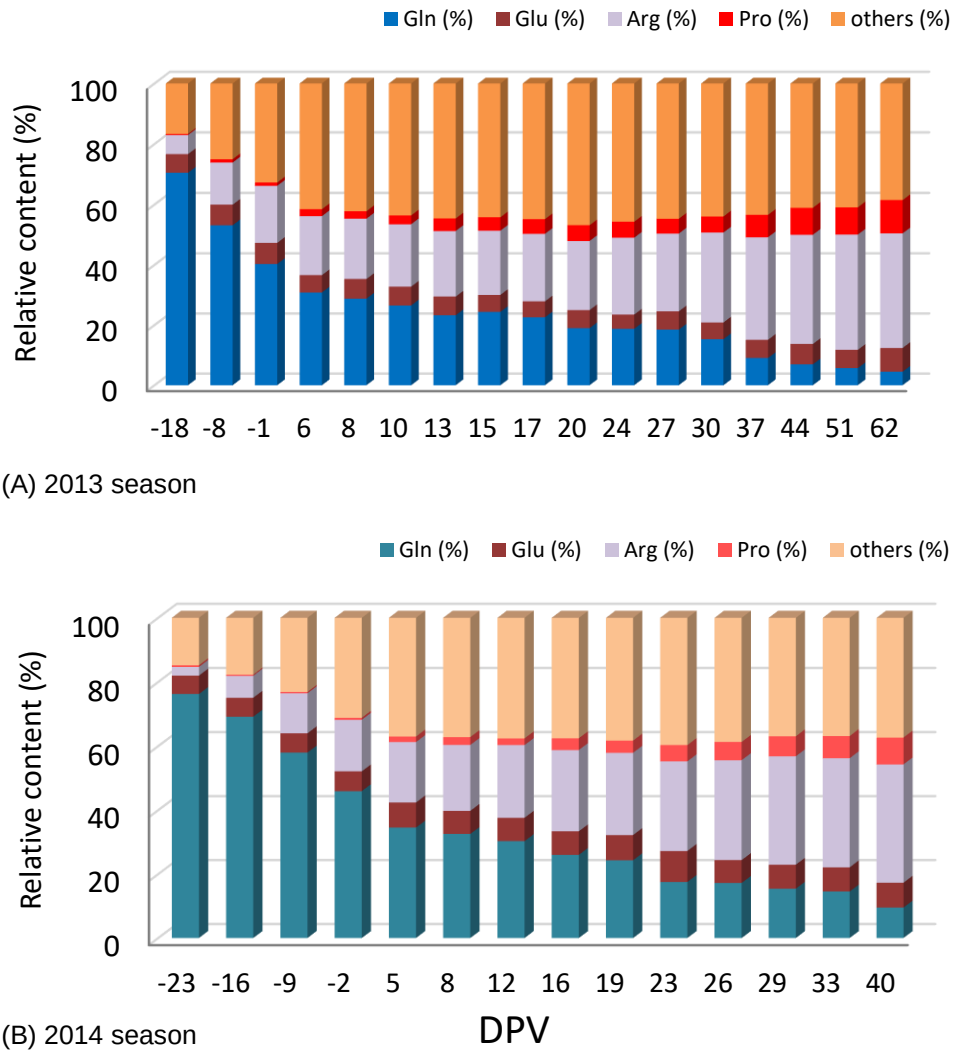


Figure 4.3 Relative content of the α-ketoglutarate amino acids

Relative proportions of the α-ketoglutarate amino acids (as percentages of total amino acid concentrations) in Sauvignon blanc grapes during berry development. CANOPY control samples are shown from the (A) 2013 season and (B) 2014 season. Sampling times are represented with respect to veraison (DPV, days postveraison). Each data point is the mean ± SEM (n = 3).

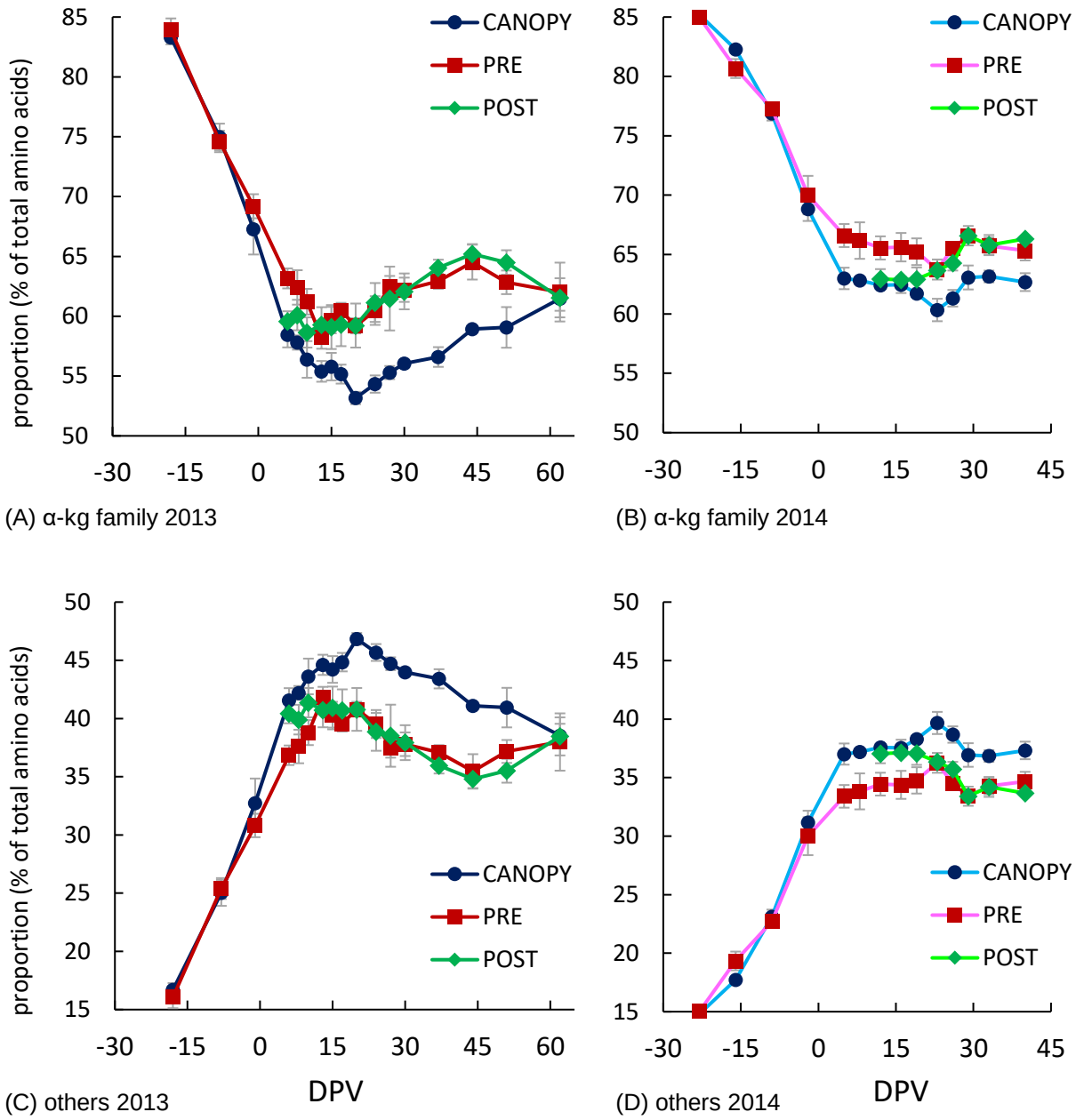


Figure 4.4 Qualitative effects of basal leaf removal on amino acid accumulation.

Proportions of the α-ketoglutarate (α-kg) amino acids in Sauvignon blanc grapes during berry development during the (A) 2013 season and (B) 2014 season. Proportions of the other (others, excluding the α-ketoglutarate family) amino acids in Sauvignon blanc grapes during berry development during the (C) 2013 season and (D) 2014 season. Relative proportions were calculated as percentages of total amino acid concentrations, with respect appropriate treatment, CANOPY control and (PRE and POST) leaf removal treatments. Sampling times are represented with respect to veraison (DPV, days postveraison). Each data point is the mean ± SEM (n = 3).

4.4.2 Glutamine and glutamate

Glutamine is easily the most abundant amino acid preveraison, being 70% of total amino acids at -18 DPV in 2013 and 76% at -23 DPV in 2014. The glutamine concentrations in both years then decrease significantly (*P*-value <.001) through development to be only 4.5% and 9.5% of total amino acids at harvest in 2013 (62 DPV) and 2014 (40 DPV) respectively (Figure 4.5 and Table 4.3). The fastest rate of decrease occurs preveraison in both seasons. In 2013, leading up to veraison (-1 DPV), glutamine concentration in the CANOPY control decreases 52% in just 17 days and a further 37% at a considerably slower rate over 63 days to 62 DPV. Similarly in 2014, the glutamine concentration decreases 35% preveraison from -23 DPV to veraison (-2 DPV) followed by a slower decline of 47% over the remaining 42 days of sampling. Both preveraison and postveraison leaf removal significantly reduced accumulation of glutamine at sampling time points during the postveraison ripening stage of development (Figure 4.5 and Appendix 3). Although, by harvest in both seasons, there was no differences between the CANOPY control, PRE and POST treatments.

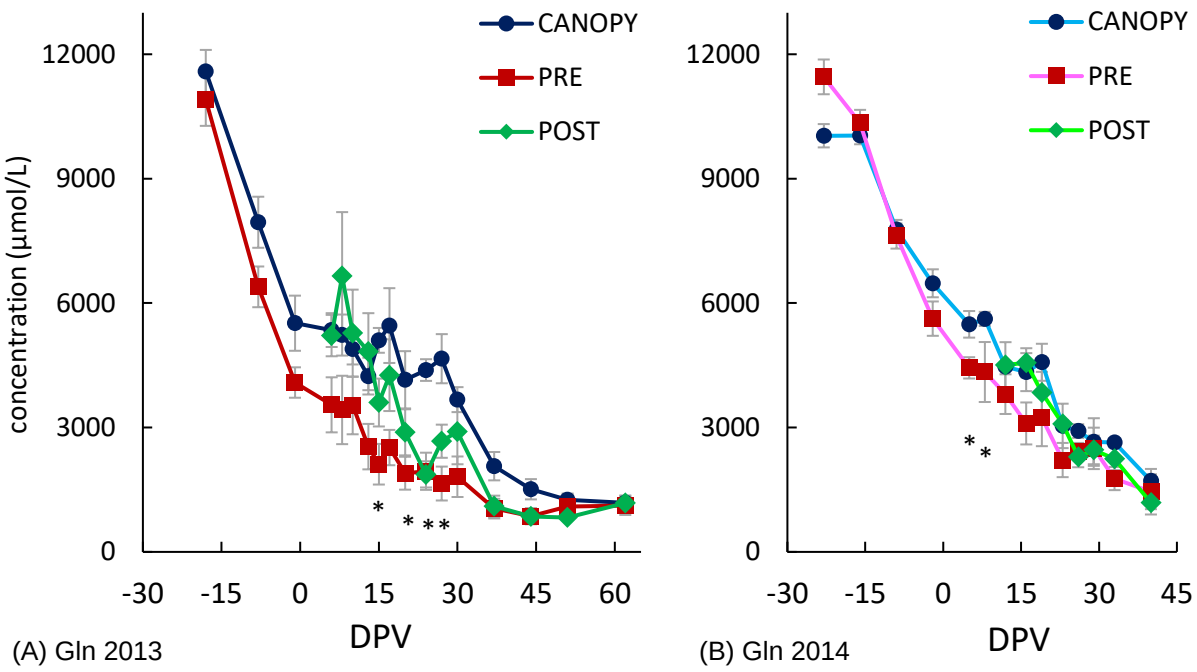


Figure 4.5 Effect of basal leaf removal on glutamine concentrations.

Glutamine (Gln) concentrations in Sauvignon blanc grapes during berry development, comparing CANOPY controls and (PRE and POST) leaf removal treatments. (A) 2013 season and (B) 2014 season. Sampling times are represented with respect to veraison (DPV, days postveraison). Each data point is the mean ± SEM (n = 3). Asterisks (*) indicate statistical differences of treatments using ANOVA (*lsd* at 5% level, see Appendix 3).

Preveraison concentrations of glutamate are considerably lower than glutamine and in general, concentrations of glutamate are reasonably consistent throughout development in both seasons (Figure 4.6 and Table 4.3). In comparison to glutamine, glutamate is present at only 6% of total amino acids at both -18 DPV in 2013, and at -23 DPV in 2014. Nevertheless, glutamate is the second most abundant amino acid preveraison. Despite its relative consistent levels, in both seasons glutamate does slowly accumulate through development to be 8% (at 62 DPV) and 7% (at 40 DPV) of total amino acids in 2013 and 2014 respectively (*P*-value <.001). Preveraison and postveraison leaf removal significantly reduced accumulation of glutamate at some sampling time points during development (Figure 4.6 and Appendix 3). By harvest (62 DPV) in 2013, there was no differences between the CANOPY control, PRE and POST treatments. In 2014, glutamate concentrations were significantly reduced in the PRE and POST treatments at 40 DPV.

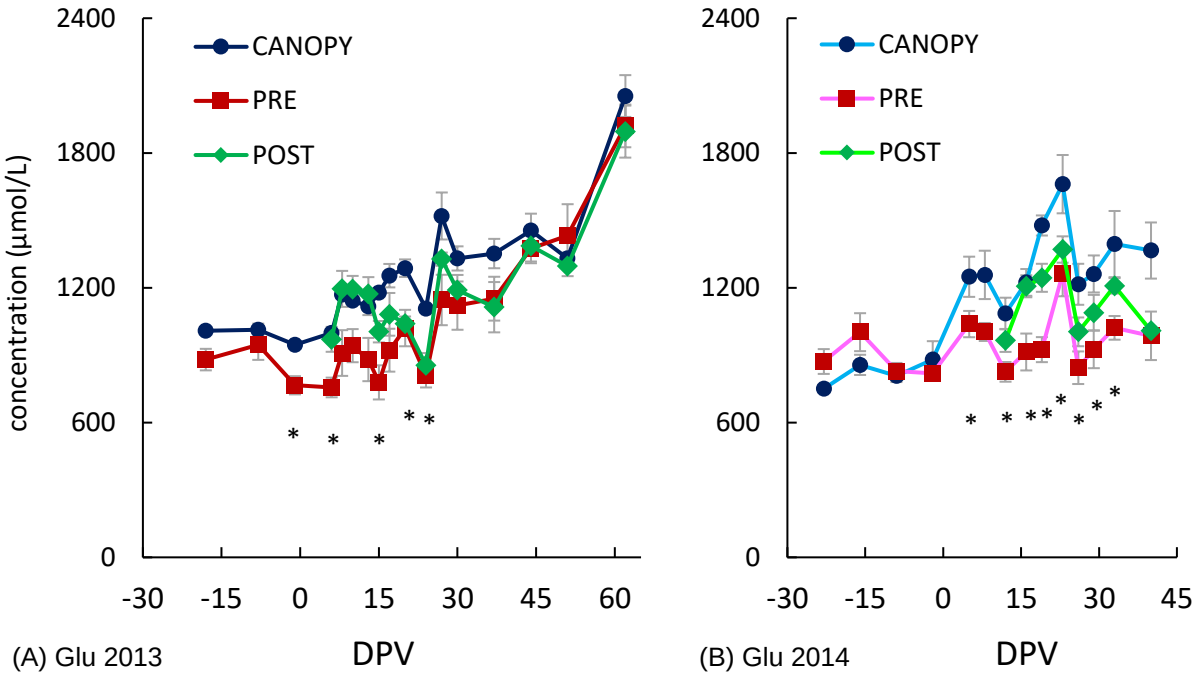


Figure 4.6 Effect of basal leaf removal on glutamate concentrations.

Glutamate (Glu) concentrations in Sauvignon blanc grapes during berry development, comparing CANOPY controls and (PRE and POST) leaf removal treatments. (A) 2013 season and (B) 2014 season. Sampling times are represented with respect to veraison (DPV, days postveraison). Each data point is the mean ± SEM (n = 3). Asterisks (*) indicate statistical differences of treatments using ANOVA (*lsd* at 5% level, see Appendix 3).

4.4.3 Arginine and proline

Dramatic changes in the content of arginine and proline occur during Sauvignon blanc berry development in both seasons. Accumulation of arginine clearly begins preveraison and increases consistently through development (*P*-value <.001). So consistent is the rate of accumulation of arginine during development, that a simple linear regression of the CANOPY control data in 2013 yields an R^2 value of 0.98, and in 2014, and R^2 value of 0.99 (data not shown). Arginine is also the predominant amino acid at harvest, at 38% of total amino acid concentration in 2013 (62 DPV) and 37% in 2014 (40 DPV) (Table 4.3). With respect to the CANOPY control, preveraison leaf removal in 2013 significantly reduced arginine accumulation at time points from veraison (-1 DPV) through mid-ripening stages to harvest (62 DPV) (Figure 4.7). In the PRE treatment, arginine was also the only individual amino acid to be statistically significantly reduced at harvest (Appendix 3). Postveraison leaf removal in 2013, significantly reduced arginine accumulation at certain time points from 20 DPV to 62 DPV, also being significantly reduced at harvest. Like 2013, preveraison leaf removal in 2014 significantly reduced arginine accumulation at postveraison time points from 16 DPV to 33 DPV. In 2014, postveraison leaf removal had no effect on arginine accumulation.

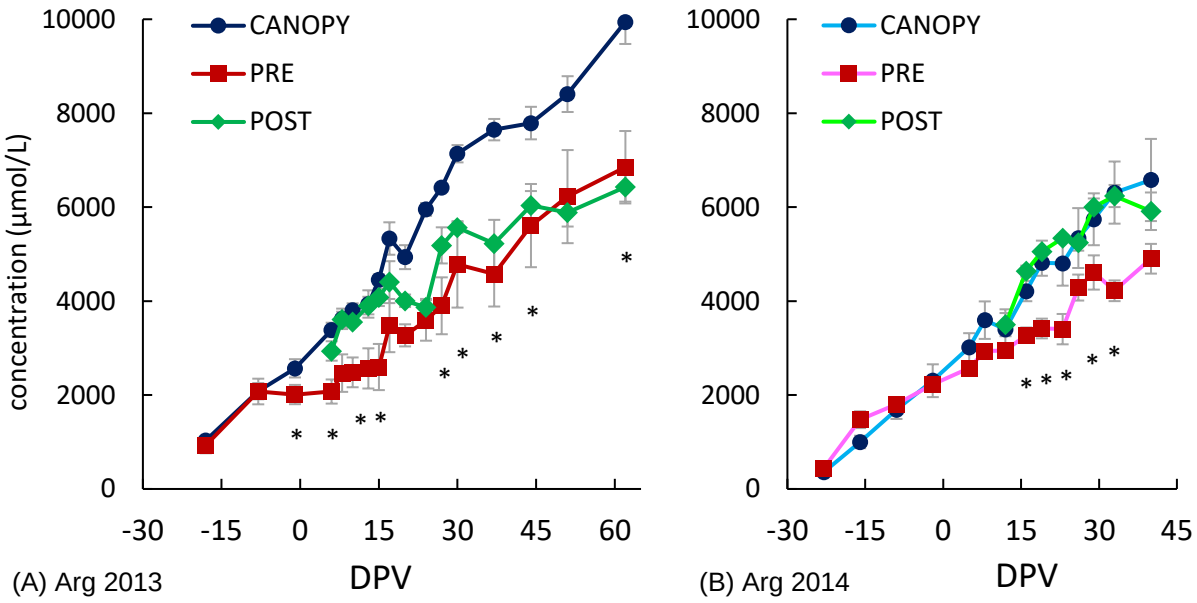


Figure 4.7 Effect of basal leaf removal on arginine concentrations.

Arginine (Arg) concentrations in Sauvignon blanc grapes during berry development, comparing CANOPY controls and (PRE and POST) leaf removal treatments. (A) 2013 season and (B) 2014 season. Sampling times are represented with respect to veraison (DPV, days postveraison). Each data point is the mean ± SEM (n = 3). Asterisks (*) indicate statistical differences of treatments using ANOVA (*lsd* at 5% level, see Appendix 3).

Proline is initially at very low levels preveraison in both seasons and then increases substantially (*P*-value <.001) after veraison through mid-ripening to harvest (Figure 4.8 and Appendix 3). At -18 DPV in 2013 and -23 DPV in 2014 proline concentrations are 0.4% and 0.5% of total amino acids respectively, and at veraison, 1.1% and 0.6% respectively. By 62 DPV and 40 DPV in 2013 and 2014 respectively, proline concentrations contribute 11% and 8.4% of total amino acids (Table 4.3). In 2013, proline accumulation is reduced after veraison with the PRE and POST leaf removal treatments. However, from 24 DPV, PRE and POST proline concentrations rebound to become equivalent to the control by 30 DPV and continue to accumulate at the same rate as the control through to 62 DPV. This is a different profile to 2014 and our published profile of proline (Gregan et al. 2012), where proline concentrations in leaf removal treatments continue to be reduced compared to CANOPY controls through development right up to harvest.

Seasonal effects clearly contributed differences with respect to total amino acid accumulation (see Chapter 4.2.3). Comparing between seasons, at veraison in each season, arginine and proline concentrations were very similar. At the final sampling time point in 2014 of 40 DPV, arginine and proline concentrations in control berries were 6578 µmol/L and 1505 µmol/L respectively. At the equivalent time point the previous season in 2013 (37 DPV), arginine concentrations in control berries were 17% higher at 7650 µmol/L and proline concentrations were 12% higher at 1682 µmol/L.

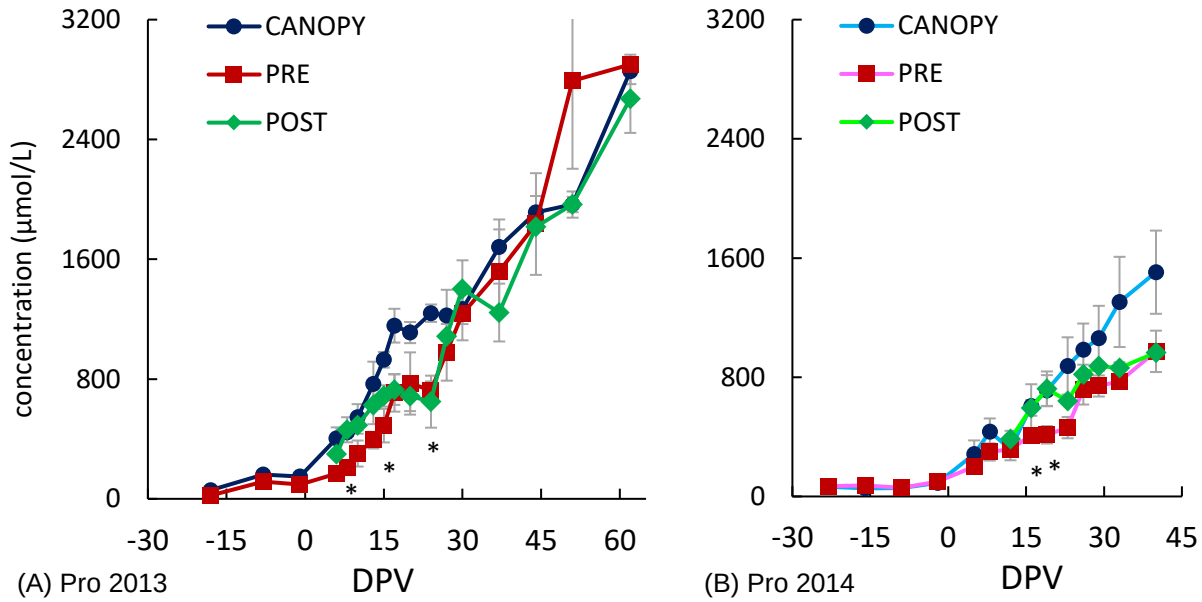


Figure 4.8 Effect of basal leaf removal on proline concentrations.

Proline (Pro) concentrations in Sauvignon blanc grapes during berry development, comparing CANOPY controls and (PRE and POST) leaf removal treatments. (A) 2013 season and (B) 2014 season. Sampling times are represented with respect to veraison (DPV, days postveraison). Each data point is the mean ± SEM (n = 3). Asterisks (*) indicate statistical differences of treatments using ANOVA (*lsd* at 5% level, see Appendix 3).

4.5 The aspartate metabolic family of amino acids

The aspartate/oxaloacetate family of amino acids is composed of aspartate, asparagine, threonine, isoleucine, methionine and lysine. Aspartate is the biosynthetic precursor of asparagine, threonine, methionine and lysine. Threonine then gives rise to isoleucine. Aspartate is also an essential cofactor in the arginine biosynthetic pathway (see Chapter 1.8.2).

4.5.1 Aspartate and asparagine

In both seasons, aspartate shows a minimal developmental response, maintaining relatively consistent concentrations through the veraison transition, mid-ripening and late development stages (Figure 4.9 and Appendix 3). Leaf removal in the PRE treatments significantly reduces aspartate concentrations at time points from veraison (-1 DPV) to 17 DPV in 2013 and from 15 DPV to 27 DPV in 2014. Later in development in both seasons, there was no difference in aspartate concentrations between the control, PRE and POST treatments. At the final sampling time points of 62 DPV in 2013 and 40 DPV in 2014, aspartate concentrations were 1% and 3.5% of total amino acids respectively (Table 4.3).

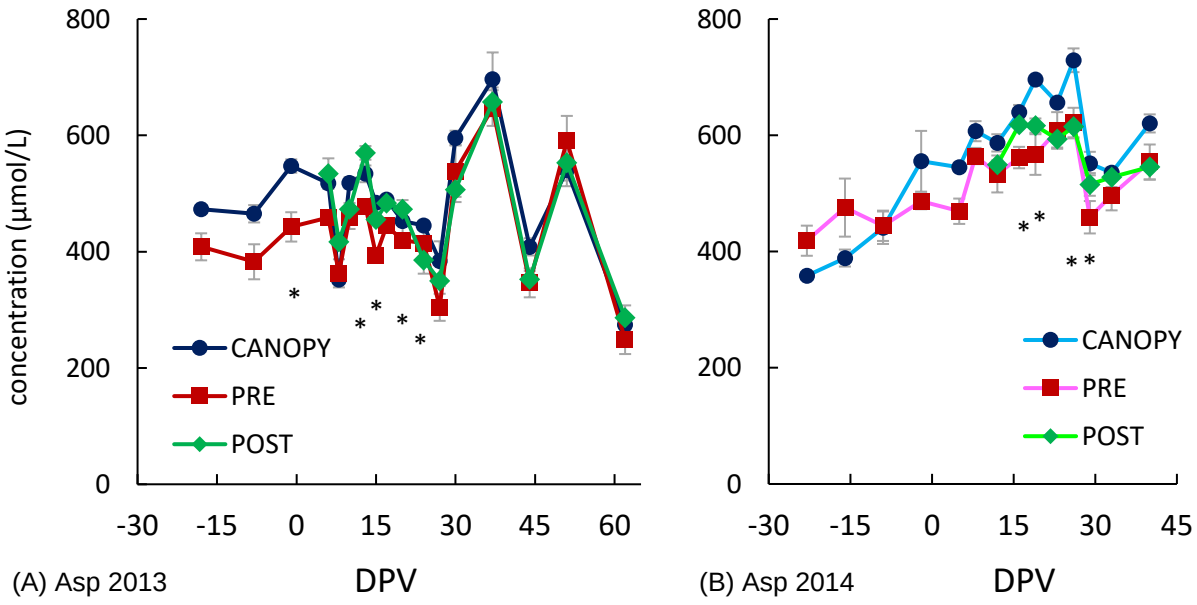


Figure 4.9 Effect of basal leaf removal on aspartate concentrations.

Aspartate (Asp) concentrations in Sauvignon blanc grapes during berry development, comparing CANOPY controls and (PRE and POST) leaf removal treatments. (A) 2013 season and (B) 2014 season. Sampling times are represented with respect to veraison (DPV, days postveraison). Each data point is the mean ± SEM (n = 3). Asterisks (*) indicate statistical differences of treatments using ANOVA (*lsd* at 5% level, see Appendix 3).

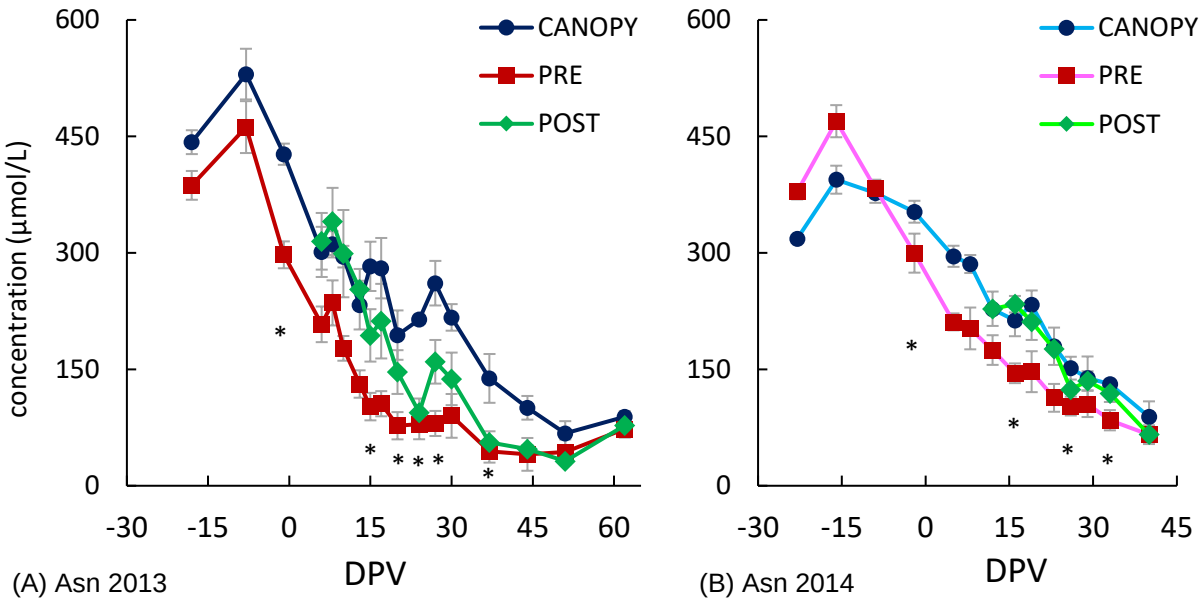


Figure 4.10 Effect of basal leaf removal on asparagine concentrations.

Asparagine (Asn) concentrations in Sauvignon blanc grapes during berry development, comparing CANOPY controls and (PRE and POST) leaf removal treatments. (A) 2013 season and (B) 2014 season. Sampling times are represented with respect to veraison (DPV, days postveraison). Each data point is the mean ± SEM (n = 3). Asterisks (*) indicate statistical differences of treatments using ANOVA (*lsd* at 5% level, see Appendix 3).

Asparagine showed a developmental profile very similar to glutamine and along with glutamine, was the only individual amino acid profile to show concentrations subsequently decreasing through development (Figure 4.10) (*P*-value <.001). In both seasons, asparagine concentrations decrease consistently to be only 0.3% of total amino acids at 62 DPV in 2013, and 0.5% at 40 DPV in 2014. Leaf removal in PRE treatments significantly reduces asparagine accumulation at time points postveraison in both seasons (Appendix 3). In 2013, postveraison leaf removal also reduced asparagine concentrations at mid-ripening time points, 24 DPV and 27 DPV. Postveraison leaf removal in 2014 had no effect on asparagine concentrations.

There were no seasonal effects on accumulation of aspartate and asparagine, concentrations of both essentially equivalent at comparable developmental time points.

4.5.2 Threonine, isoleucine and methionine

Threonine, isoleucine and methionine all show similar profiles of accumulation through development despite large differences in their concentrations. They are all initially at low levels preveraison and then increase substantially after veraison, peaking at mid-ripening time points in both seasons (Figure 4.11, Figure 4.12 and Figure 4.13).

Threonine is the fifth most abundant amino acid with concentrations only slightly less than proline at harvest. Preveraison at -18 DPV in 2013 and -23 DPV in 2014, threonine concentrations are 1.4% and 1% of total amino acids respectively, and at veraison, 3.8% and 3.2% respectively. By 62 DPV and 40 DPV in 2013 and 2014 respectively, threonine concentrations contribute 9% and 8% of total amino acids. In 2013, isoleucine and methionine contributed 3.3% and 0.6% respectively, of total amino acids at 62 DPV. In 2014, isoleucine and methionine were 1.7% and 0.5% of total amino acids at 40 DPV (Table 4.3). Preveraison leaf removal significantly reduces accumulation of threonine, isoleucine and methionine at postveraison time points in both seasons. Postveraison leaf removal significantly reduced accumulation of methionine and isoleucine in both seasons and threonine in 2013 (Appendix 3). Similarly to arginine, seasonal effects contribute differences in the accumulation of threonine, isoleucine and methionine. Comparisons between seasons at veraison shows similar concentrations. From veraison onwards, control levels of threonine, isoleucine and methionine in 2013, continue to accumulate at considerably higher concentrations than observed in 2014 at equivalent developmental time points.

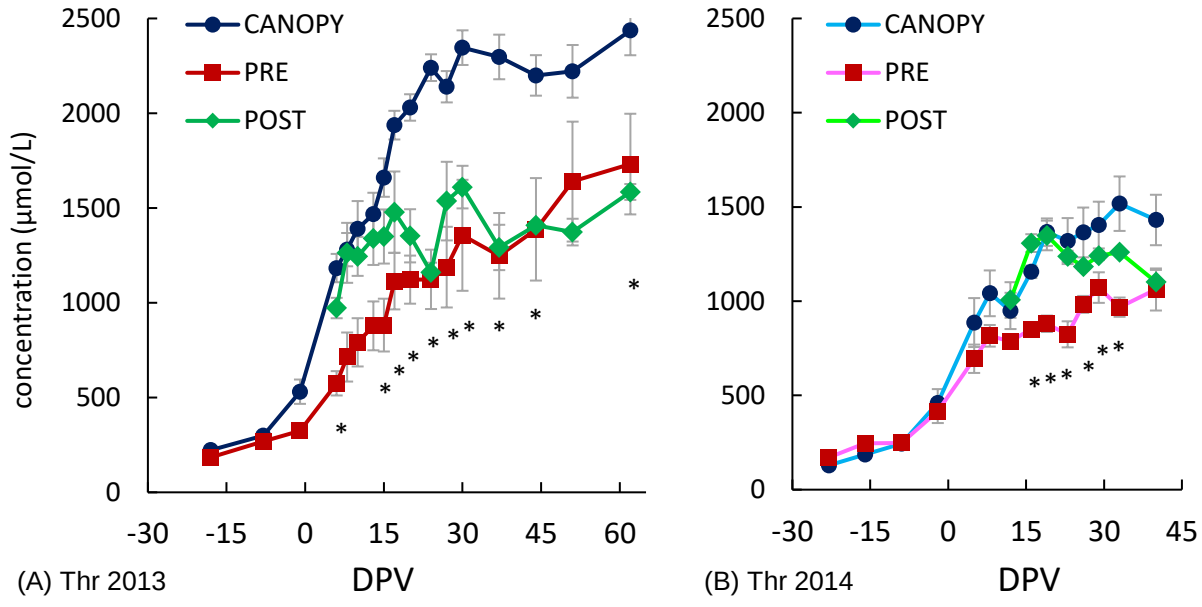


Figure 4.11 Effect of basal leaf removal on threonine concentrations.

Threonine (Thr) concentrations in Sauvignon blanc grapes during berry development, comparing CANOPY controls and (PRE and POST) leaf removal treatments. (A) 2013 season and (B) 2014 season. Sampling times are represented with respect to veraison (DPV, days postveraison). Each data point is the mean ± SEM (n = 3). Asterisks (*) indicate statistical differences of treatments using ANOVA (*lsd* at 5% level, see Appendix 3).

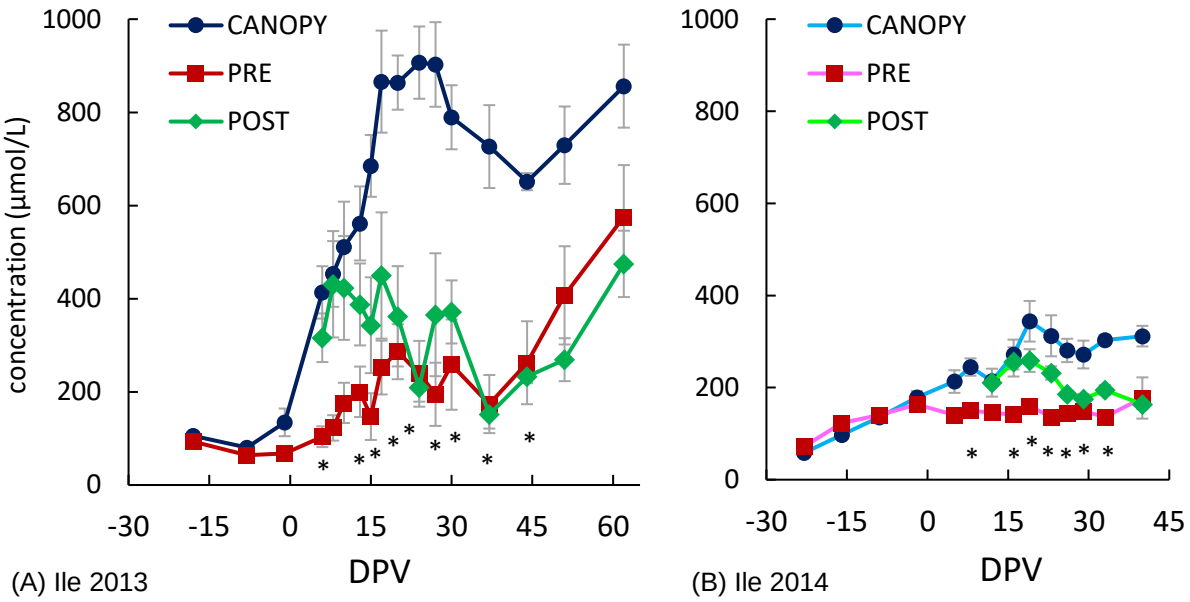


Figure 4.12 Effect of basal leaf removal on isoleucine concentrations.

Isoleucine (Ile) concentrations in Sauvignon blanc grapes during berry development, comparing CANOPY controls and (PRE and POST) leaf removal treatments. (A) 2013 season and (B) 2014 season. Sampling times are represented with respect to veraison (DPV, days postveraison). Each data point is the mean ± SEM (n = 3). Asterisks (*) indicate statistical differences of treatments using ANOVA (*lsd* at 5% level, see Appendix 3).

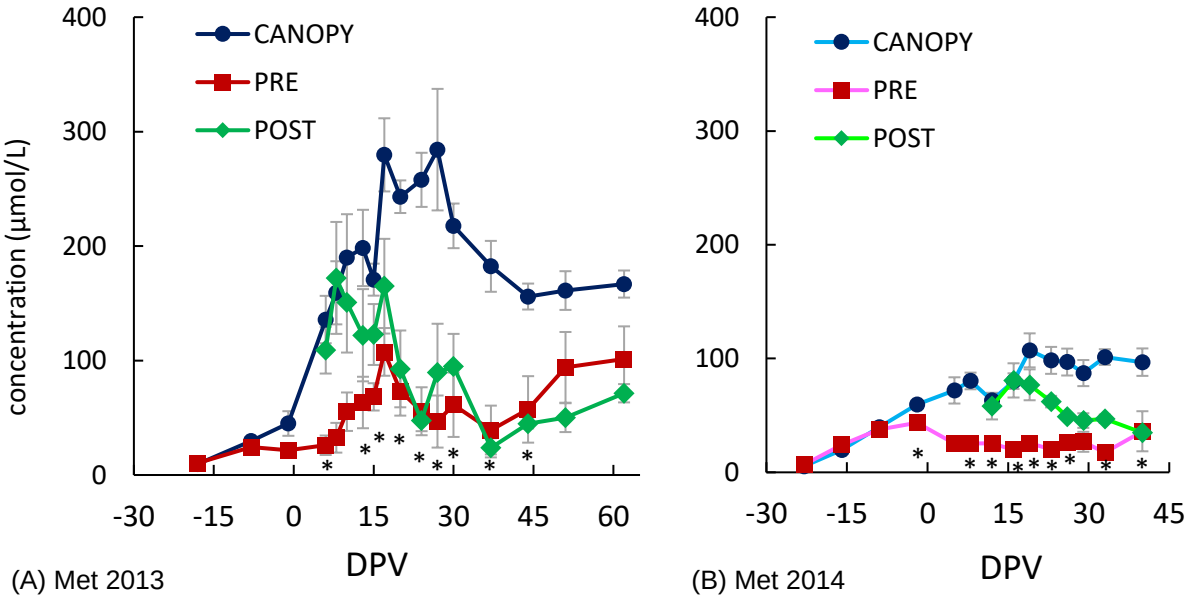


Figure 4.13 Effect of basal leaf removal on methionine concentrations.

Methionine (Met) concentrations in Sauvignon blanc grapes during berry development, comparing CANOPY controls and (PRE and POST) leaf removal treatments. (A) 2013 season and (B) 2014 season. Sampling times are represented with respect to veraison (DPV, days postveraison). Each data point is the mean ± SEM (n = 3). Asterisks (*) indicate statistical differences of treatments using ANOVA (*lsd* at 5% level, see Appendix 3).

4.5.3 Lysine

Lysine is present at relatively low concentrations through development in both seasons (Figure 4.14 and Table 4.3). By 62 DPV in 2013 and 40 DPV in 2014, lysine concentrations contribute only 0.9% and 0.2% of total amino acids respectively.

In 2013, leaf removal in the PRE and POST treatments, significantly reduce concentrations of lysine at the postveraison time points 27-37 DPV and 30-51 DPV respectively (Appendix 3). There is no effect of leaf removal earlier in development up until 24 DPV, lysine concentrations being equivalent to the control treatment. Lysine concentrations in the PRE treatment in 2014 are slightly higher than the control preveraison and through the veraison transition, although these slight increases were not statistically significant at any time point.

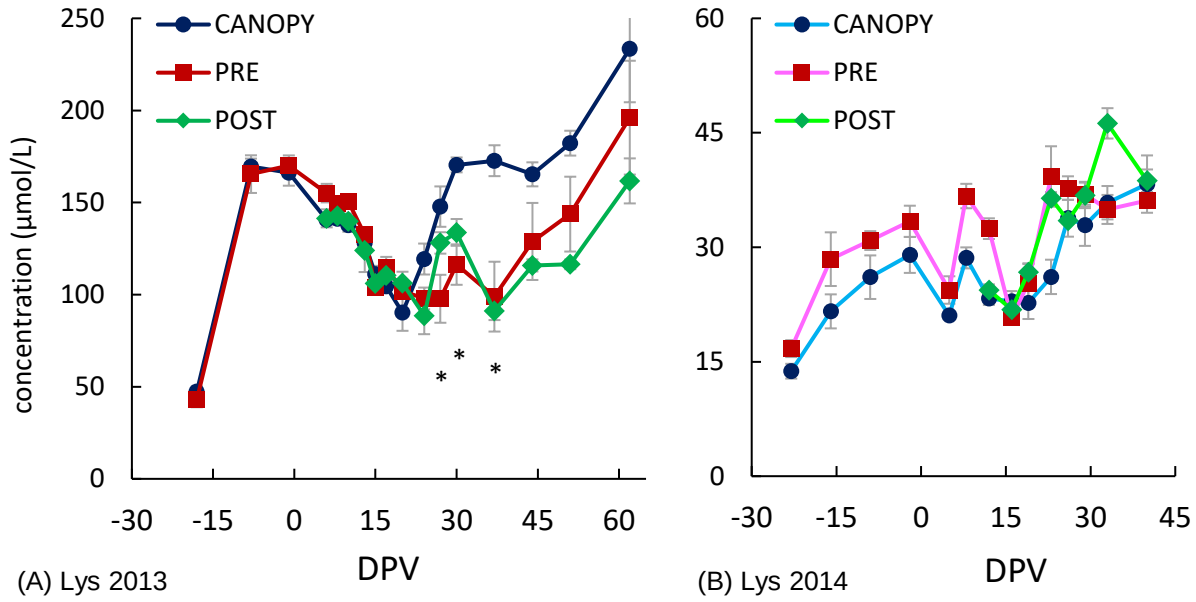


Figure 4.14 Effect of basal leaf removal on lysine concentrations.

Lysine (Lys) concentrations in Sauvignon blanc grapes during berry development, comparing CANOPY controls and (PRE and POST) leaf removal treatments. (A) 2013 season and (B) 2014 season. Sampling times are represented with respect to veraison (DPV, days postveraison). Each data point is the mean ± SEM (n = 3). Asterisks (*) indicate statistical differences of treatments using ANOVA (*lsd* at 5% level, see Appendix 3).

4.6 The pyruvate metabolic family of amino acids

Pyruvate is the end result of glycolysis and supplies energy to cells through the TCA cycle. Additionally it is the starting precursor for several amino acids. Reactions beginning with either one or two molecules of pyruvate lead to the biosynthesis of leucine, alanine and valine.

4.6.1 Leucine, alanine and valine

The individual profiles of accumulation through development of these three biosynthetically related amino acids, are remarkably similar to each other as observed in both seasons (Figure 4.15, Figure 4.16 and Figure 4.17). In 2013, leucine, alanine and valine control concentrations increase substantially after veraison, all three peaking at 27 DPV and finishing less than this peak at harvest (62 DPV). Alanine concentrations in particular, decrease 50% from 1978 µmol/L at 27 DPV to 1087 µmol/L at 62 DPV. Leucine, alanine and valine concentrations in the control treatments contributed 5%, 4% and 3.2% respectively, of total amino acids at 62 DPV (Table 4.3). With respect to the control, preveraison leaf removal in 2013 significantly reduced leucine, alanine and valine accumulation at time points from veraison (-1 DPV) through mid-ripening stages to 44 DPV. Postveraison leaf removal in 2013, significantly reduced accumulation at time points from 15 DPV to 51 DPV (Appendix 3).

As seen with other amino acids, seasonal effects contributed differences in the accumulation of leucine and valine. Comparisons between the 2013 and 2014 seasons at veraison shows similar concentrations. After veraison, control levels of leucine and valine in 2013, continue to accumulate to significantly higher concentrations than observed in 2014 at equivalent developmental time points. Leucine, alanine and valine concentrations contributed 2.2%, 8% and 2% respectively, of total amino acids at 40 DPV. Nevertheless, preveraison leaf removal in 2014 still significantly reduced leucine, alanine and valine accumulation at time points after veraison. Postveraison leaf removal significantly decreased leucine and valine concentrations at time points after veraison (Appendix 3).

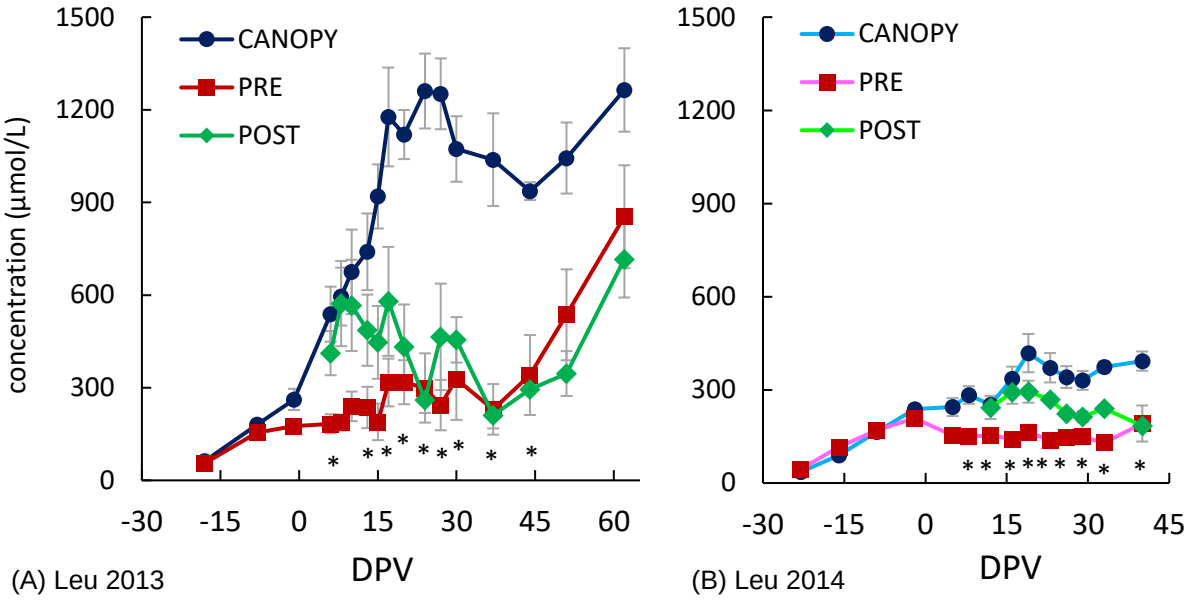


Figure 4.15 Effect of basal leaf removal on leucine concentrations.

Leucine (Leu) concentrations in Sauvignon blanc grapes during berry development, comparing CANOPY controls and (PRE and POST) leaf removal treatments. (A) 2013 season and (B) 2014 season. Sampling times are represented with respect to veraison (DPV, days postveraison). Each data point is the mean ± SEM (n = 3). Asterisks (*) indicate statistical differences of treatments using ANOVA (*lsd* at 5% level, see Appendix 3).

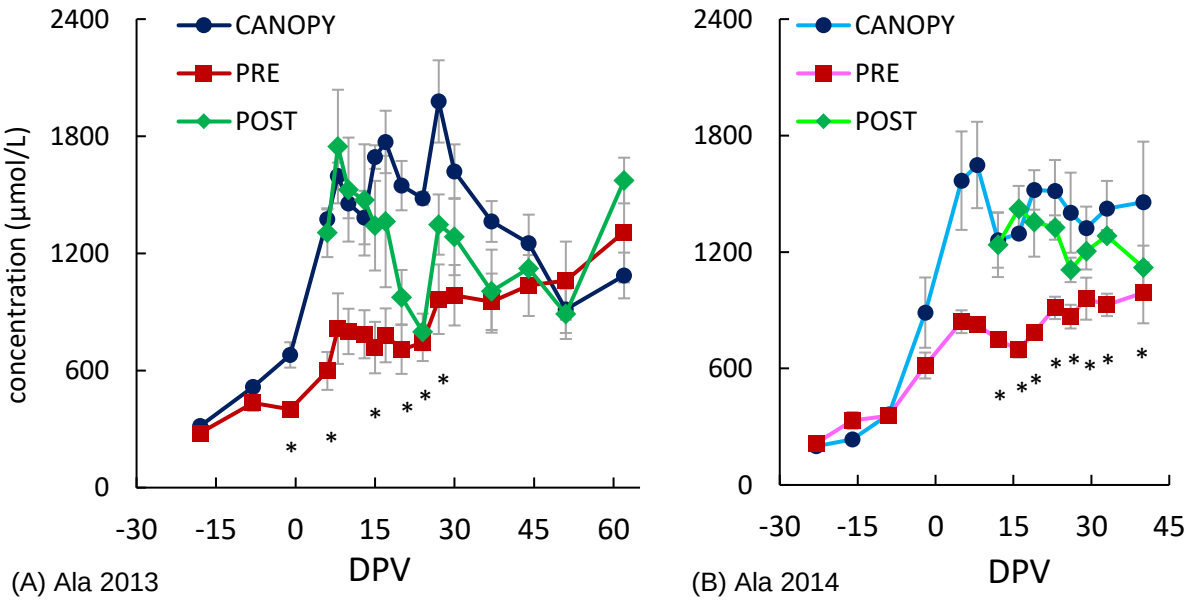


Figure 4.16 Effect of basal leaf removal on alanine concentrations.

Alanine (Ala) concentrations in Sauvignon blanc grapes during berry development, comparing CANOPY controls and (PRE and POST) leaf removal treatments. (A) 2013 season and (B) 2014 season. Sampling times are represented with respect to veraison (DPV, days postveraison). Each data point is the mean ± SEM (n = 3). Asterisks (*) indicate statistical differences of treatments using ANOVA (*lsd* at 5% level, see Appendix 3).

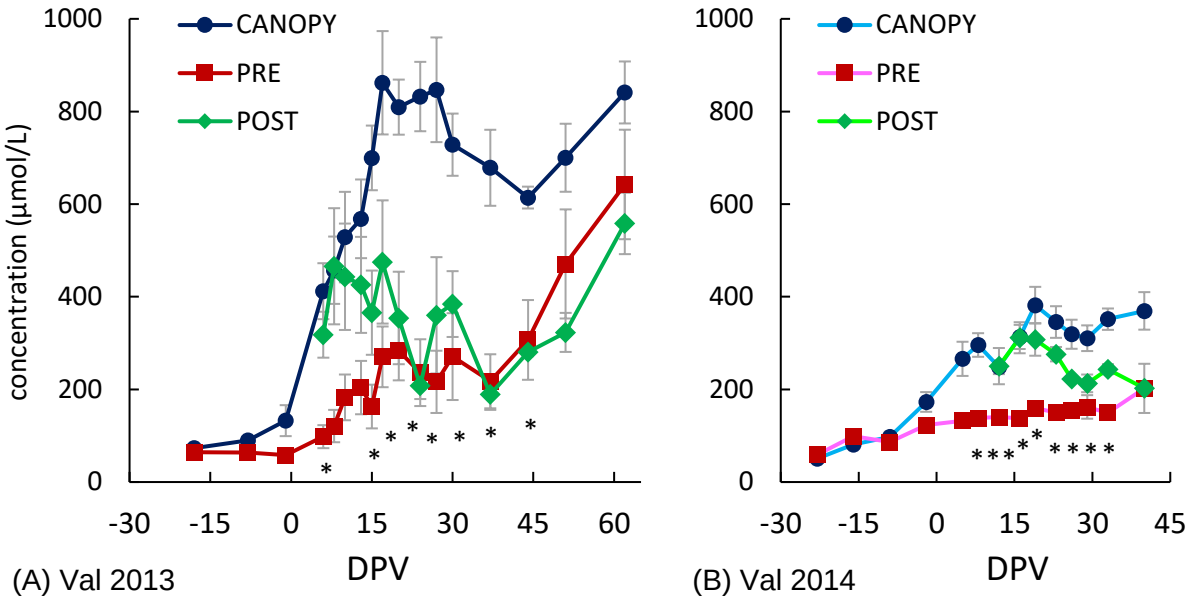


Figure 4.17 Effect of basal leaf removal on valine concentrations.

Valine (Val) concentrations in Sauvignon blanc grapes during berry development, comparing CANOPY controls and (PRE and POST) leaf removal treatments. (A) 2013 season and (B) 2014 season. Sampling times are represented with respect to veraison (DPV, days postveraison). Each data point is the mean ± SEM (n = 3). Asterisks (*) indicate statistical differences of treatments using ANOVA (*lsd* at 5% level, see Appendix 3).

4.7 The aromatic metabolic family of amino acids

The shikimate pathway is a complex series of metabolic reactions in plants responsible for the biosynthesis of the aromatic amino acids, phenylalanine, tryptophan and tyrosine. The precursors for this pathway are the common metabolic intermediates phosphoenolpyruvate (glycolysis) and erythrose-4-phosphate (pentose phosphate pathway). The conversion of phenylalanine to cinnamic acid via the enzyme phenylalanine ammonia-lyase, is the starting reaction in grapes for the biosynthesis of a large number of compounds important for wine quality, including flavonols, anthocyanins and other polyphenols (Jackson 2014).

4.7.1 Phenylalanine and tryptophan

The accumulation of phenylalanine and tryptophan concentrations through development had a similar profile to each other as observed in both seasons (Figure 4.18 and Figure 4.19). In the CANOPY control treatments, phenylalanine and tryptophan concentrations increase substantially after veraison in both seasons. In 2013, accumulation of both is greatest between 17-24 DPV. From 24 DPV, phenylalanine goes through a period of decline, before going through another accumulation to be equivalent with peak concentrations at 62 DPV. Tryptophan also declines from 24 DPV and finishes at 20% less than peak concentrations at 62 DPV. Phenylalanine and tryptophan peak at 19 DPV in 2014, with concentrations of both being at equivalent levels at the final sampling at 40 DPV.

Both preveraison and postveraison leaf removal significantly reduces phenylalanine and tryptophan concentrations at postveraison time points in both seasons (Appendix 3). At the final sampling time points of 62 DPV in 2013 and 40 DPV in 2014, the PRE and POST treatments were statistically indistinguishable. Additionally, phenylalanine was present in berries at a lower proportions of total amino acids with respect to basal leaf removal treatments, compared to the CANOPY controls. This qualitative effect of basal leaf removal on phenylalanine is opposite to the α-ketoglutarate amino acids and is interesting due to phenylalanine being a principal precursor for many secondary metabolites and is presented in Appendix 5.

There is a effect of season with respect to phenylalanine and tryptophan concentrations, showing differences in their accumulation through development. In 2013, both phenylalanine and tryptophan accumulate to higher concentrations than observed in 2014 at equivalent developmental time points.

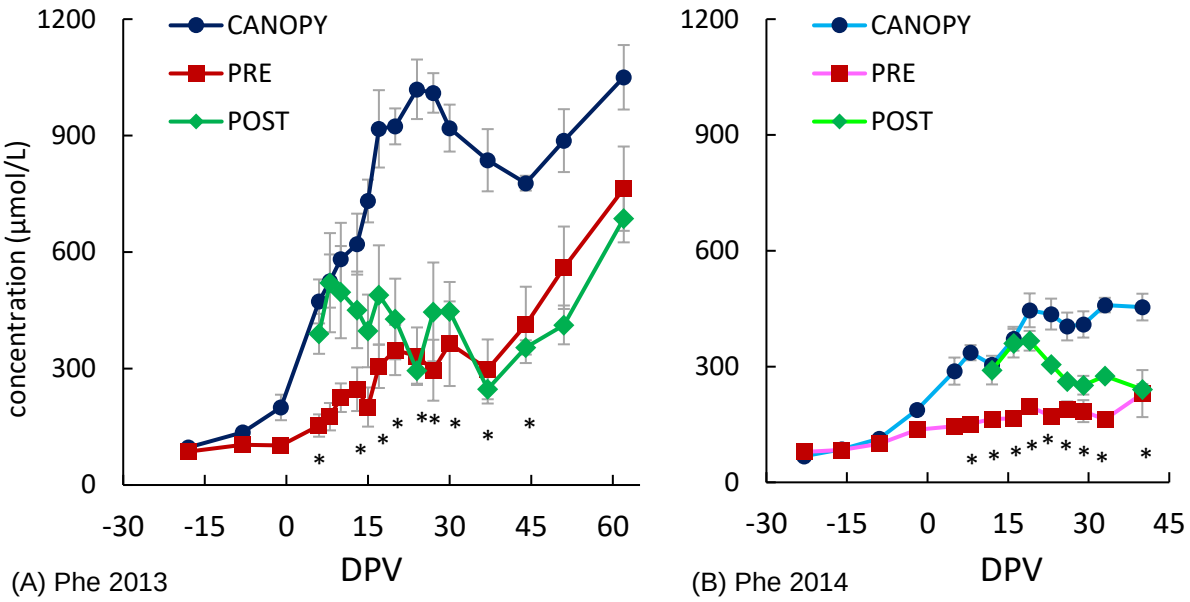


Figure 4.18 Effect of basal leaf removal on phenylalanine concentrations.

Phenylalanine (Phe) concentrations in Sauvignon blanc grapes during berry development, comparing CANOPY controls and (PRE and POST) leaf removal treatments. (A) 2013 season and (B) 2014 season. Sampling times are represented with respect to veraison (DPV, days postveraison). Each data point is the mean ± SEM (n = 3). Asterisks (*) indicate statistical differences of treatments using ANOVA (*lsd* at 5% level, see Appendix 3).

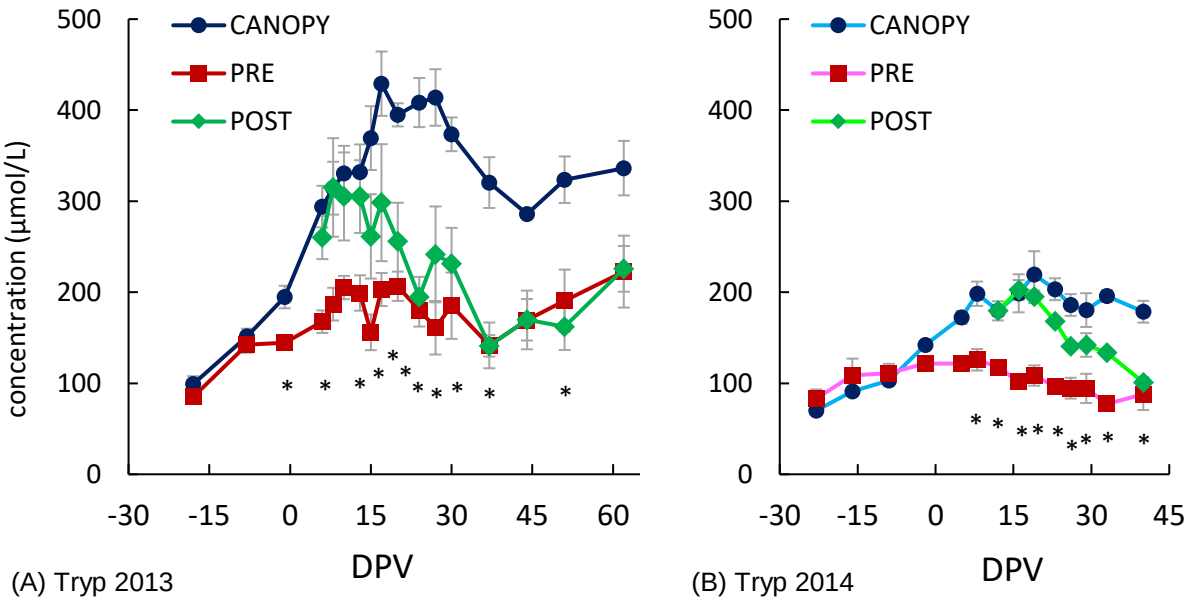


Figure 4.19 Effect of basal leaf removal on tryptophan concentrations.

Tryptophan (Tryp) concentrations in Sauvignon blanc grapes during berry development, comparing CANOPY controls and (PRE and POST) leaf removal treatments. (A) 2013 season and (B) 2014 season. Sampling times are represented with respect to veraison (DPV, days postveraison). Each data point is the mean ± SEM (n = 3). Asterisks (*) indicate statistical differences of treatments using ANOVA (*lsd* at 5% level, see Appendix 3).

4.7.2 Tyrosine

Generally, tyrosine is present at low levels throughout development, contributing only 0.7% and 0.8% of total amino acids at 62 DPV in 2013 and 40 DPV in 2014 respectively (Table 4.3). Conversely to phenylalanine and tryptophan, tyrosine concentrations increase the most preveraison, with CANOPY controls, PRE and POST treatment levels peaking just after veraison (6 DPV in 2013 and 8 DPV in 2014) (Figure 4.20). From their peaks at 6 DPV in 2013, the control, PRE and POST treatments all go through a period of decline, before going through another accumulation to be equivalent with peak concentrations at 62 DPV. In 2014, all treatments go through a similar postveraison decline to be lower than their peak concentration by 40 DPV.

Preveraison and postveraison leaf removal significantly reduced tyrosine concentrations at certain time points after veraison in both seasons (Appendix 3). Similarly to phenylalanine and tryptophan, at the final sampling time points in 2013 and 2014, tyrosine concentrations in the PRE and POST treatments were equivalent. There were no seasonal effects on accumulation of tyrosine, concentrations being essentially equivalent at comparable developmental time points.

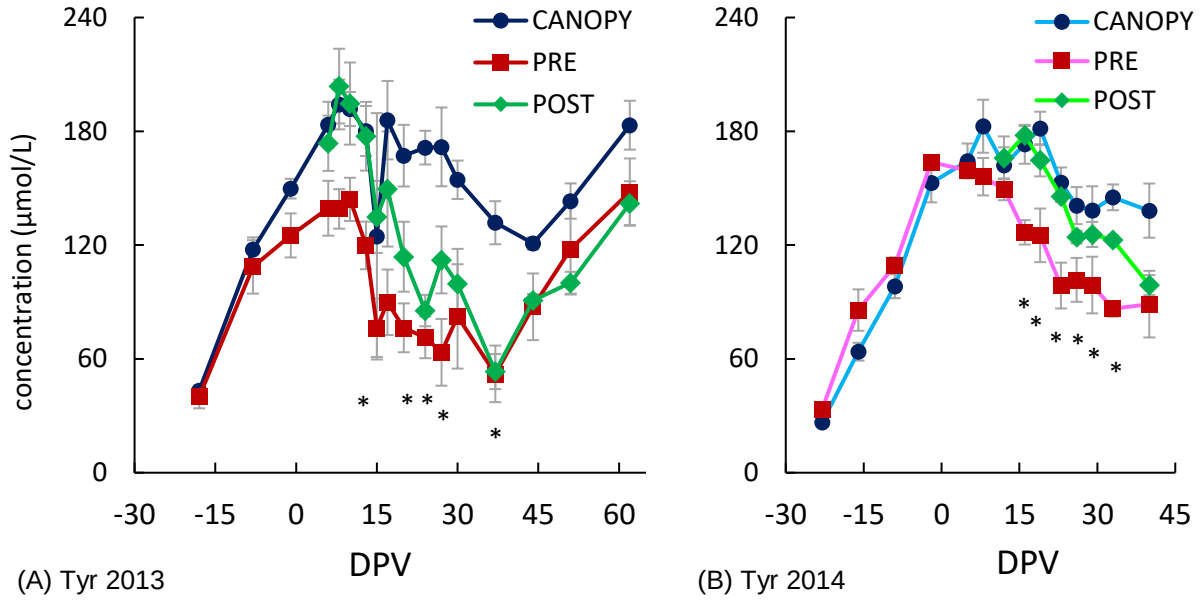


Figure 4.20 Effect of basal leaf removal on tyrosine concentrations.

Tyrosine (Tyr) concentrations in Sauvignon blanc grapes during berry development, comparing CANOPY controls and (PRE and POST) leaf removal treatments. (A) 2013 season and (B) 2014 season. Sampling times are represented with respect to veraison (DPV, days postveraison). Each data point is the mean ± SEM (n = 3). Asterisks (*) indicate statistical differences of treatments using ANOVA (*lsd* at 5% level, see Appendix 3).

4.8 Histidine

Histidine is present at low levels throughout development, contributing only 0.9% and 1.2% of total amino acids at 62 DPV in 2013 and 40 DPV in 2014 respectively (Table 4.3). Similarly to tyrosine, histidine concentrations in the control treatment in 2013, peak just after veraison at 6 DPV. In 2014, histidine concentrations are relatively consistent through veraison, having a sharp increase between 23-33 DPV and subsequently declining slightly at 40 DPV (Figure 4.21).

Preveraison and postveraison leaf removal significantly reduced histidine concentrations at some time points after veraison in both seasons. At the final sampling time points in 2013 and 2014, there was no significant differences between histidine concentrations in the control, PRE and POST treatments (Appendix 3). The seasonal differences observed between the 2013 and 2014 CANOPY control concentrations was not statistically significant.

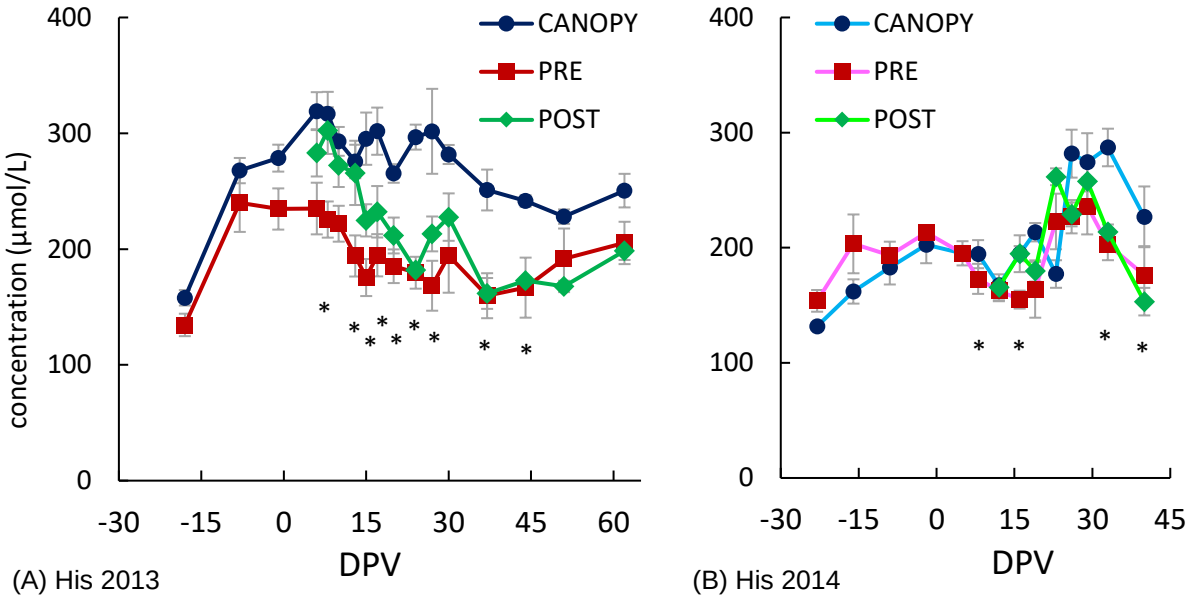


Figure 4.21 Effect of basal leaf removal on histidine concentrations.

Histidine (His) concentrations in Sauvignon blanc grapes during berry development, comparing CANOPY controls and (PRE and POST) leaf removal treatments. (A) 2013 season and (B) 2014 season. Sampling times are represented with respect to veraison (DPV, days postveraison). Each data point is the mean ± SEM (n = 3). Asterisks (*) indicate statistical differences of treatments using ANOVA (*lsd* at 5% level, see Appendix 3).

4.9 The 3-phosphoglycerate metabolic family of amino acids

4.9.1 Serine

In the control treatments, accumulation of serine begins preveraison but nevertheless, increases sharply at veraison and peaks at 17-27 DPV in 2013 and 19-23 DPV in 2014 (Figure 4.22). In both seasons, serine concentrations in control treatments decline to be slightly less than their peaks at the final sampling time points of 62 DPV in 2013 and 40 DPV in 2014. Serine contributes 3.5% and 4.1% of total amino acids at 62 DPV in 2013 and 40 DPV in 2014 respectively (Table 4.3).

Preveraison leaf removal has the effect on reducing accumulation of serine through development in both seasons. Serine concentrations in the PRE treatment are significantly decreased at both preveraison and postveraison time points in 2013, and at postveraison time points in 2014 (Appendix 3). Postveraison leaf removal significantly reduced serine concentrations at some time points after veraison in both seasons. In 2013, serine concentrations in the PRE treatment rebound from 44 DPV to be equivalent with the control at 62 DPV. There were no seasonal effects on accumulation of serine, concentrations were similar at equivalent developmental time points.

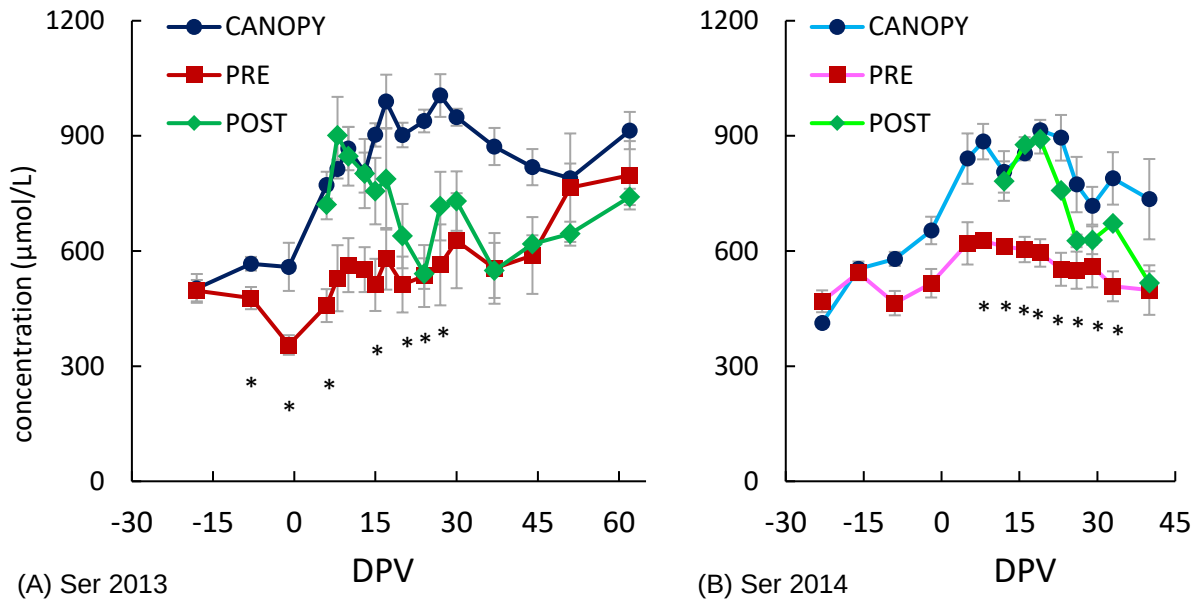


Figure 4.22 Effect of basal leaf removal on serine concentrations.

Serine (Ser) concentrations in Sauvignon blanc grapes during berry development, comparing CANOPY controls and (PRE and POST) leaf removal treatments. (A) 2013 season and (B) 2014 season. Sampling times are represented with respect to veraison (DPV, days postveraison). Each data point is the mean ± SEM (n = 3). Asterisks (*) indicate statistical differences of treatments using ANOVA (*lsd* at 5% level, see Appendix 3).

4.9.2 Glycine

In general, glycine is present at low levels throughout development, concentrations in the control treatments, contributing only 0.3% and 0.2% of total amino acids at 62 DPV in 2013 and 40 DPV in 2014 respectively (Table 4.3). Despite low levels of glycine, it does go through a period of accumulation and subsequent decline through the veraison transition in both seasons (Figure 4.23). Interestingly, preveraison leaf removal significantly increases concentrations of glycine at time points during this period. In both seasons, PRE treatment concentrations then decline postveraison to be equivalent with control concentrations by 15 DPV in 2013 and 19 DPV in 2014. From 20 DPV in 2013, PRE and POST concentrations of glycine are actually then decreased compared to the control treatment, although these decreases were not statistically significant. Postveraison leaf removal has no significant effects on glycine concentrations in 2014. At the final sampling time points in 2013 and 2014, there was no differences between glycine concentrations in the control, PRE and POST treatments (Appendix 3). No seasonal effects were observed on accumulation of glycine, concentrations were comparable at equivalent developmental time points.

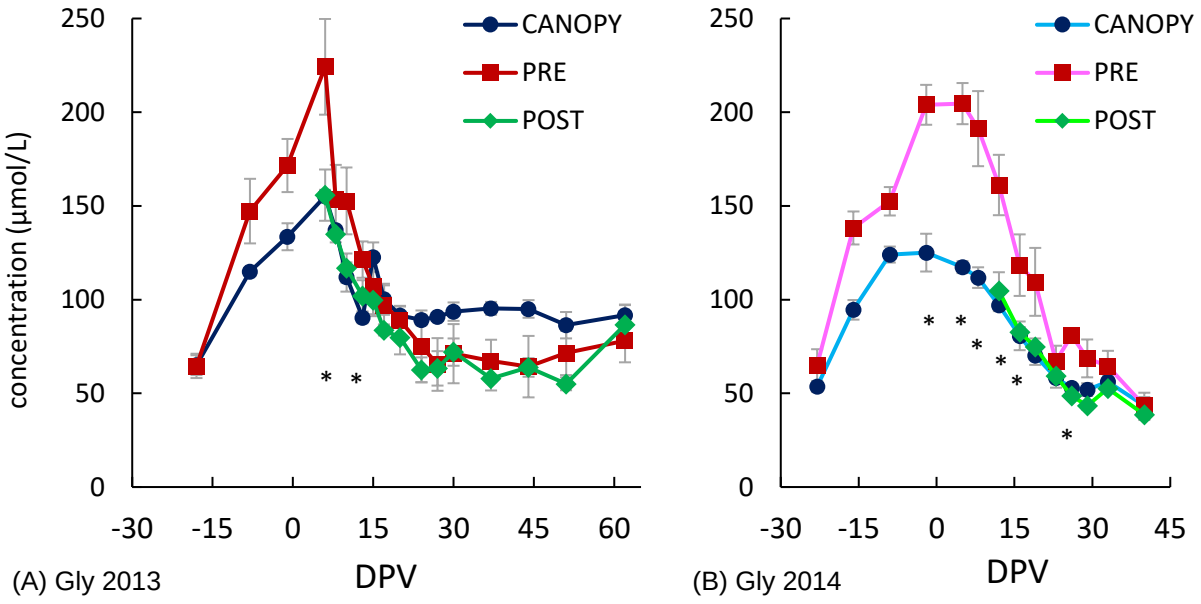


Figure 4.23 Effect of basal leaf removal on glycine concentrations.

Glycine (Gly) concentrations in Sauvignon blanc grapes during berry development, comparing CANOPY controls and (PRE and POST) leaf removal treatments. (A) 2013 season and (B) 2014 season. Sampling times are represented with respect to veraison (DPV, days postveraison). Each data point is the mean ± SEM (n = 3). Asterisks (*) indicate statistical differences of treatments using ANOVA (*lsd* at 5% level, see Appendix 3).

4.9.3 Cysteine

Cysteine was not detectable at any time point in 2013. In 2014, cysteine concentrations were detected at a number of time points through development, although measurements were inconsistent and at baseline detection thresholds. Therefore, the cysteine results are not shown and were not included in any subsequent analysis.

4.10 Conclusions

Basal leaf removal has a significant effect on amino acid accumulation in Sauvignon blanc grape berries. The quantitative effect of leaf removal is a reduction of total amino acid concentrations in the grapes, which is reflected at the level of amino acid families and individual amino acids. In addition, leaf removal also has a qualitative effect on the proportions of some individual amino acids. For example, the α-ketoglutarate amino acid concentrations are reduced in the PRE and POST leaf removal treatments, but as a proportion of total amino acids (with respect to their treatment) are higher than CANOPY control samples. The results therefore demonstrate, that the α-ketoglutarate amino acids occupy a greater percentage with the total amino acid pool in samples from the basal leaf removal treatments.

This suggests that basal leaf removal can impact regulation of the amino acid biochemical pathways. In addition to quantitatively decreasing concentrations, this in turn can modify the various proportions of amino acids that accumulate in the grape berries, with respect to the different leaf removal treatments. The results presented in this chapter suggest the prospect of a number of mechanisms that could affect both quantitative and qualitative characteristics of amino acid metabolism, the significance of which is investigated further in Chapter 5 and Chapter 6.

Chapter 5
Gene transcript analysis of the α-ketoglutarate amino acids in Sauvignon blanc grapes

5.1 Introduction

Amino acid concentrations clearly change significantly through development in Sauvignon blanc grapes. This observation is reflected in total amino acid concentrations in the berry, differential accumulation at the level of amino acid families and within families, individual amino acids (Chapter 4). In addition, both preveraison and postveraison basal leaf removal significantly reduces total amino acid accumulation throughout development. Individual amino acids also have differential responses to leaf removal. Aside from the quantitative effect of basal leaf removal on amino acid concentrations, leaf removal also had a qualitative effect on some individual amino acids, modifying their proportions of accumulation in the grape berries.

The important observations presented in Chapter 4 suggest the possibility of a number of mechanisms that could affect both quantitative and qualitative aspects of amino acid metabolism, such as a reduction in the import of glutamine (due to a decrease in the proximal leaf area), transcriptional changes of biosynthetic genes and/or allosteric regulation of associated enzymes.

The results presented in this chapter examine the mechanisms of amino acid accumulation and responses of Sauvignon blanc berries to basal leaf removal, by investigating transcriptional changes of genes involved in amino acid metabolic pathways. A range of genes involved in different aspects of amino acid assimilation, biosynthesis, catabolism and regulation were investigated. The focus was genetic regulation of the α-ketoglutarate family of amino acids (glutamine, glutamate, arginine and proline), being the family that contains the predominant concentrations of amino acids in the berry and have both quantitative and qualitative aspects in their response to leaf removal. Using the Nanostring nCounter system (Geiss et al. 2008), expression of genes involved in nitrogen assimilation (through glutamine and glutamate) and arginine/proline biosynthetic and degradation pathways were quantified through development. We also investigated genes reputedly involved in proline accumulation as a result of a potentially active alternative pathway via arginine and ornithine intermediates (see Chapter 1.9).

5.2 RNA quality control

5.2.1 Bioanalyzer and Fragment Analyzer analysis

Total RNA was extracted from Sauvignon blanc grapes as described in Chapter 2.6. The concentration and quality of all RNA samples were initially determined by fluorometric and spectrophotometric analysis. Additionally, to ensure that the purified RNA samples were suitable for nCounter analysis, the integrity of a "representative" subset of 24 RNA samples were also assessed using an Agilent Bioanalyzer. The "representative" samples chosen were spread across the developmental stages from the 2013 and 2014 vineyards experiments. The Bioanalyzer generates an RNA Integrity Number (RIN), which is a measure of the integrity and level of degradation of an RNA sample. The RIN value is used to determine if the RNA can be used for sensitive downstream applications such as sequencing or nCounter analysis. The RIN scale ranges from 1 (degraded) to 10 (intact) and in general, with plant RNA, RIN values approaching 8 or greater are preferred. Results from the Bioanalyzer runs indicated that the "representative" subset purified RNA samples extracted from Sauvignon blanc grapes were suitable for nCounter analysis (data not shown).

Additionally, all 198 samples sent to NZGL for nCounter analysis were first analysed using their Fragment Analyzer. This was an in-house quality control provided by NZGL to ensure the RNA sample integrity. Like the Bioanalyzer, the Fragment Analyzer generates a RIN score to evaluate RNA quality and assess each sample for any potential degradation, which could lead to an unsuccessful nCounter analysis. Out of the 198 samples submitted to NZGL, only one RNA sample showed degradation and was omitted from the nCounter assays and subsequent analyses (Figure 5.1).

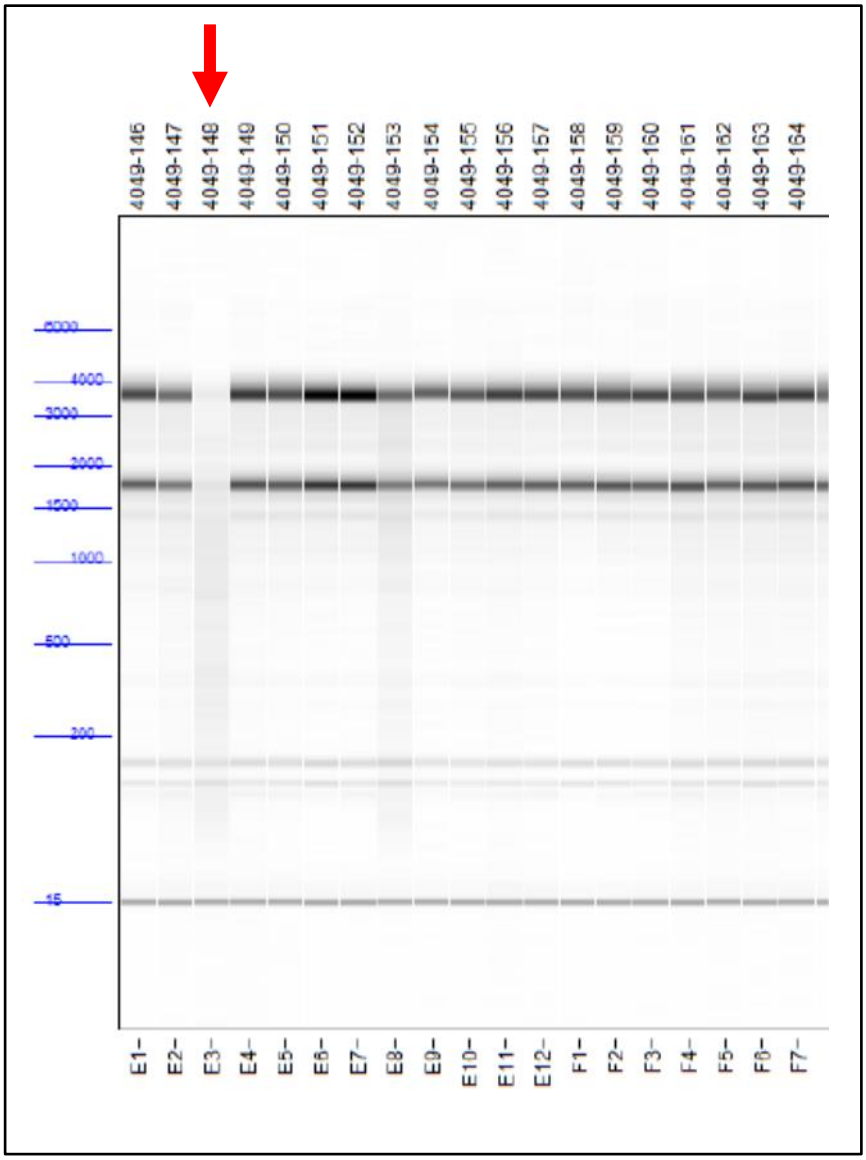


Figure 5.1 Fragment Analyzer analysis of RNA from Sauvignon blanc grapes.

A subset of RNA samples are shown that were analysed with the Fragment Analyzer. Samples were run prior to nCounter analysis to ensure the integrity of the RNA. Only one sample (from the entire batch of 198) was deemed degraded and not suitable for further analysis (indicated with the red arrow). All other RNA samples passed NZGL's quality control and continued through the nCounter analysis. (Picture of partial gel image was supplied courtesy of NZGL).

5.2.2 Determination of reference genes and a positive gene control for nCounter analysis

To confirm the suitability for genetic analysis, cDNA samples synthesised from purified total RNA were first analysed using qPCR. qPCR was initially used to investigate two important considerations before analysis of RNA for nCounter analysis:

- The appropriate reference (housekeeping) genes to use for qPCR and nCounter analysis.
- An appropriate positive control for nCounter analysis.

Reference gene selection can have a considerable impact on normalised gene expression data in qPCR experiments (Reid et al. 2006). Therefore, before their use in qPCR and/or nCounter analyses, it was important to evaluate reference gene stability. Specifically in the nCounter analysis, reference gene normalisation is used to adjust transcript counts relative to transcripts (reference genes) that are not expected to vary between samples or replicates. Reference gene normalisation assumes that the reference target sequences recognised by their probes for nCounter are consistent in their expression levels. Therefore, the choice of reference genes to include for the nCounter normalisation is a crucial part of the experimental design (Geiss et al. 2008).

It is consequently worth putting considerable effort into validating reference gene(s) chosen for normalisation prior to extensive (and expensive) experimentation. Therefore, four reference genes that had previously been identified for their relative stability in developmental studies of grape berry were initially analysed (*VvGAPDH*, *VvActin*, *VvEF-1α* and *VvSAND*) across our samples as described in Chapter 2.6. Using the geNorm software tool, *GAPDH* and *Actin* were identified as the two most stably expressed genes in berry samples across the 2013 and 2014 experiments and used as the reference genes for normalisation of data in qPCR and nCounter analysis (data not shown).

Additionally, to demonstrate the validity of our vineyard experiments, it was important to identify a transcript that could be used as a positive control for leaf removal in the nCounter analysis. *VvFLS4* expression was initially tested using qPCR analysis. Across the 2013 and 2014 experiment samples, *FLS4* expression was shown to be upregulated in the PRE and POST leaf removal treatments, compared to the CANOPY controls (data not show). Therefore, *FLS4* was included in the nCounter analysis as a positive control gene (see Chapter 5.3.2 below).

5.3 The Nanostring nCounter system

The Nanostring nCounter system allows analysis of multiple genes within a single sample. It is a highly sensitive and accurate detection method and has been validated against other quantitative methods such as qPCR (Geiss et al. 2008). We utilised this technology to simultaneously look at expression of a number of genes in related amino acid metabolic pathways. The nCounter system provides a direct readout of the amount of transcript counts in a RNA sample without any additional amplification or other steps which may introduce bias. The quantity of transcript counts the nCounter system can detect is stable across a large dynamic range, which makes this analysis technique extremely useful in determining absolute levels of expression and comparing multiple individual mRNA transcript abundance within a sample.

5.3.1 Genes/transcripts analysed

The range of genes quantified are summarised in Table 5.1 and contain a mix of characterised genes through to putative genes and uncharacterised expressed transcripts. The probe sequences used to target the transcripts are described in Appendix 2, with each probe designed to specifically target only the transcript sequence of interest.

Table 5.1 Vitis vinifera transcripts analysed using nCounter analysis.

Gene (transcript)	Gene name	Predicted function	Accession	Reference
FLS4	Vitis vinifera flavonol synthase	Flavonol biosynthesis	AB092591.1	Fujita et al. (2006)
GS1-1	Vitis vinifera glutamine synthetase1-1	Nitrogen assimilation	NM_001281246.1	Loulakakis & Roubelakis-Angelakis (1996)
GS1-2	Vitis vinifera glutamine synthetase1-2	Nitrogen assimilation	NM_001281125.1	Loulakakis & Roubelakis-Angelakis (1996)
GS1-3	Vitis vinifera glutamine synthetase1-3	Nitrogen assimilation	NM_001281175.1	Loulakakis & Roubelakis-Angelakis (1996)
NADH-GOGAT-1	Predicted: Vitis vinifera glutamate synthase 1 (NADH)	Nitrogen assimilation	XM_002267829.2	http://www.genoscope.cns.fr/vitis Jaillon et al. (2007)
NADH-GOGAT-2	Predicted: Vitis vinifera glutamate synthase (NADH)-like	Nitrogen assimilation	XM_003633822.1	http://www.genoscope.cns.fr/vitis Jaillon et al. (2007)
P5CS	Vitis vinifera pyrroline-5-carboxylate synthetase	Proline biosynthesis	NM_001281205.1	Stines et al. (1999)
P5CS1a	Predicted: Vitis vinifera pyrroline-5-carboxylate synthetase-like	Proline biosynthesis	XM_002273220.2	http://www.genoscope.cns.fr/vitis Jaillon et al. (2007)
P5CS1b	Predicted: Vitis vinifera pyrroline-5-carboxylate synthetase-like	Proline biosynthesis	VIT_15s0024g00720	http://www.genoscope.cns.fr/vitis Jaillon et al. (2007)
P5CR	Predicted: Vitis vinifera pyrroline-5-carboxylate reductase-like	Proline biosynthesis	XM_003632680.1	http://www.genoscope.cns.fr/vitis Jaillon et al. (2007)
PDH	Predicted: Vitis vinifera proline dehydrogenase-like	Proline metabolism	XM_002282733.1	http://www.genoscope.cns.fr/vitis Jaillon et al. (2007)
P5CDH	Predicted: Vitis vinifera pyrroline-5-carboxylate dehydrogenase-like	Proline metabolism	XM_002273533.1	http://www.genoscope.cns.fr/vitis Jaillon et al. (2007)
Arginase	Predicted: Vitis vinifera arginase-like	Arginine metabolism	XM_002280654.2	http://www.genoscope.cns.fr/vitis Jaillon et al. (2007)
OAT	Vitis vinifera ornithine aminotransferase	Arginine metabolism	NM_001281140.1	(Venturini et al. 2013)
SNAC2	Predicted: Vitis vinifera NAC-domain containing protein 2	Transcription factor – regulator of *OAT*	XM_002274141.4	http://www.genoscope.cns.fr/vitis Jaillon et al. (2007)

5.3.2 Flavonol synthase (*VvFLS4*) expression - a positive control for leaf removal (light exposure)

The expression of *VvFLS4* in Sauvignon blanc grapes has previously been shown to be regulated by exposure to light/UV-B radiation and has also shown to be developmentally regulated, increasing postveraison during berry ripening (Downey et al. 2003; Fujita et al. 2006; Gregan et al. 2012; Liu et al. 2015). *FLS4* was therefore included in the nCounter analysis as a positive control for leaf removal and particularly, light exposure. The use of *FLS4* as a positive control for nCounter analysis had initially been tested by qPCR, which showed *FLS4* to be upregulated in the PRE and POST leaf removal treatment samples (see Chapter 5.2.2). Therefore, it was considered that *FLS4* would be a suitable control to use because of its known developmental response and the fact it is induced by light exposure, and hence our leaf removal treatments Figure 5.2.

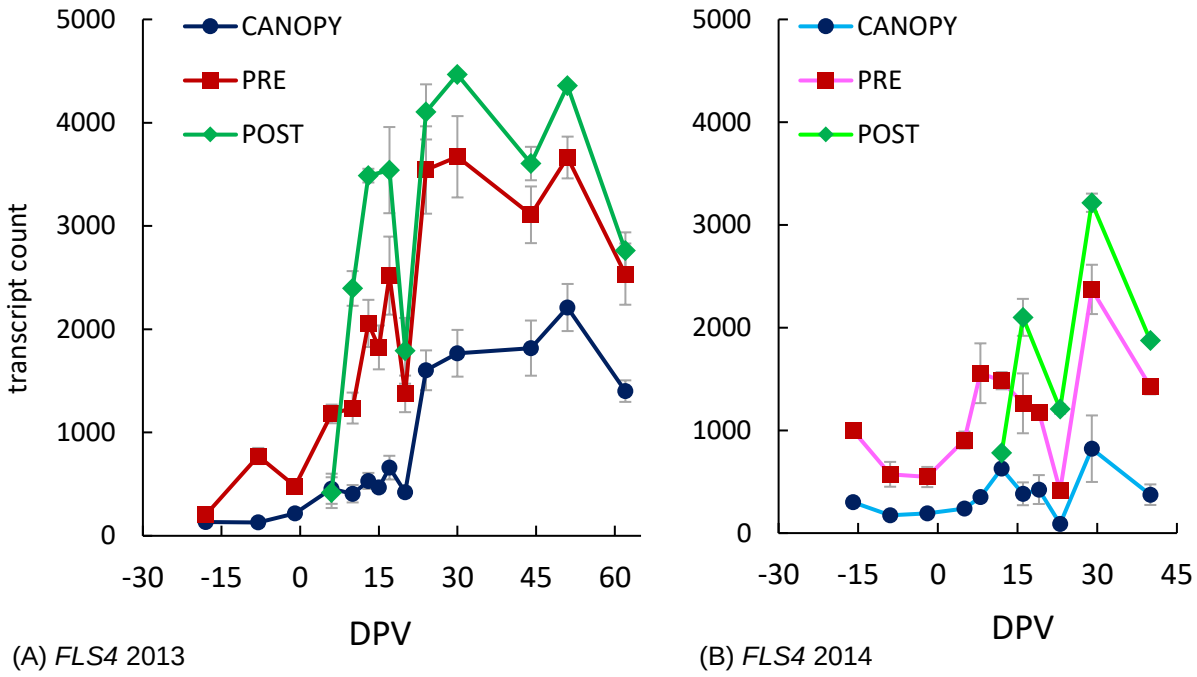


Figure 5.2 *VvFLS4* nCounter transcript analysis in Sauvignon blanc grapes.

Transcript counts are shown through berry development in the: (A) 2013 season and (B) 2014 season, comparing transcript abundance in CANOPY control samples to preveraison (PRE) and postveraison (POST) leaf removal treatments. Sampling times are represented with respect to veraison; -18 to 62 days postveraison (DPV) in 2013, and -16 to 40 DPV in 2014. Each data point is the mean ± SEM (n = 3).

FLS4 expression was found to be significantly induced in Sauvignon blanc grape berries upon leaf removal and light exposure (*P*-value <.001). Maintenance of the leaf canopy (shading of berries) resulted in berry samples with much lower transcript counts. In both seasons when compared to CANOPY control treatments, *FLS4* transcript counts were considerably higher in the PRE leaf removal treatment samples. Upon implementation of the POST leaf removal treatment at 6 DPV in 2013 and 12 DPV in 2014, *FLS4* transcript counts increase substantially in the newly exposed berries compared to the shaded CANOPY control samples.

This result indicates the success of the nCounter analysis as an experimental technique for quantifying gene expression in Sauvignon blanc grapes. It also demonstrates the consistency of the nCounter analysis between samples and treatments, with FLS4 expression showing a sharp developmental upregulation after veraison in all treatments across both seasons (at 20 DPV in 2013 and 23 DPV in 2014).

5.4 Gene expression analysis of the nitrogen metabolism

In an effort to better understand the role of the genes involved in nitrogen metabolism in grapevine, expression of several isoforms of glutamine synthetase and glutamate synthase that may potentially contribute to berry metabolism during development were examined.

5.4.1 Glutamine synthetase (*VvGS*)

The complexity of the GS gene family in grapevine is shown to be similar to well characterised examples in other plants, which show a number of isoforms differentially expressed in varied tissue types (Forde & Lea 2007; Hirel et al. 2007; Tabuchi et al. 2007). To investigate genes involved in nitrogen assimilation in grapevine, the expression patterns of three isoforms of glutamine synthetase 1 (*VvGS1-1*, *VvGS1-2* and *VvGS1-3*) were analysed in Sauvignon blanc grape berries through development. The grapevine isoforms *GS1-1*, *GS1-2* and *GS1-3* have previously had some investigation, Northern analysis demonstrating differential expression in several tissues examined (roots, shoots, leaves and berries) (Loulakakis & Roubelakis-Angelakis 1996), but no further characterisation has taken place. It was therefore of interest to examine transcript abundance of each of these isoforms in Sauvignon blanc berries to infer potential contributions to GS enzyme abundance and determine if leaf removal treatments can regulate expression.

Sequence analysis was initially performed and shows the three grapevine *GS1* isoforms encode polypeptides with high homology to cytosolic GS1 protein sequences characterised in other plants. All three isoforms of *GS1* measured were expressed in Sauvignon blanc berries throughout development, albeit at differential expression levels When compared to CANOPY control samples, no consistent response to leaf removal was seen with *GS1-1*, *GS1-2* and *GS1-3* transcript counts at any developmental stages measured (Figure 5.3, Figure 5.4 and Figure 5.5).

The transcript abundance of *GS1-1* was considerably higher than *GS1-2* and *GS1-3* in berries at all stages of development. In both seasons, *GS1-1* expression initially decreased to veraison, but nevertheless was highly expressed (11671 transcript counts at -1 DPV in 2013 and 11160 counts at -2 DPV in 2014). Expression of *GS1-1* then increases significantly postveraison in both seasons to be 4-fold higher at 62 DPV in 2013 and 2.5-fold higher at 40 DPV in 2014 (*P*-value <.001). Both *GS1-2* and *GS1-3* had much lower levels of expression than *GS1-1* and showed different developmental profiles of expression to each other and to *GS1-1*. In both seasons, *GS1-2* started at relatively high transcript abundance preveraison, then decreased 3-fold to veraison. From a low at 15 DPV in 2013, postveraison expression increased through to the final samplings. At 62 DPV in 2013, *GS1-2* transcript counts had increased to be 3-4 fold higher than their low at 15 DPV. In 2014, *GS1-2* transcript counts were reasonably static from veraison through to the final sampling. *GS1-3* expression transcript counts were considerably lower than

GS1-1 and *GS1-2*. *GS1-3* expression was highest at preveraison samplings and subsequently decreased through development.

These results suggest that expression of the *GS1-1* gene homolog is potentially a major contributor to the cytosolic *GS* isoenzymes in grape berries at all stages of development. In postveraison samples, its expression increases significantly, overlapping a time in berry development when glutamine concentrations are being depleted and glutamate is being diverted into other biochemical pathways.

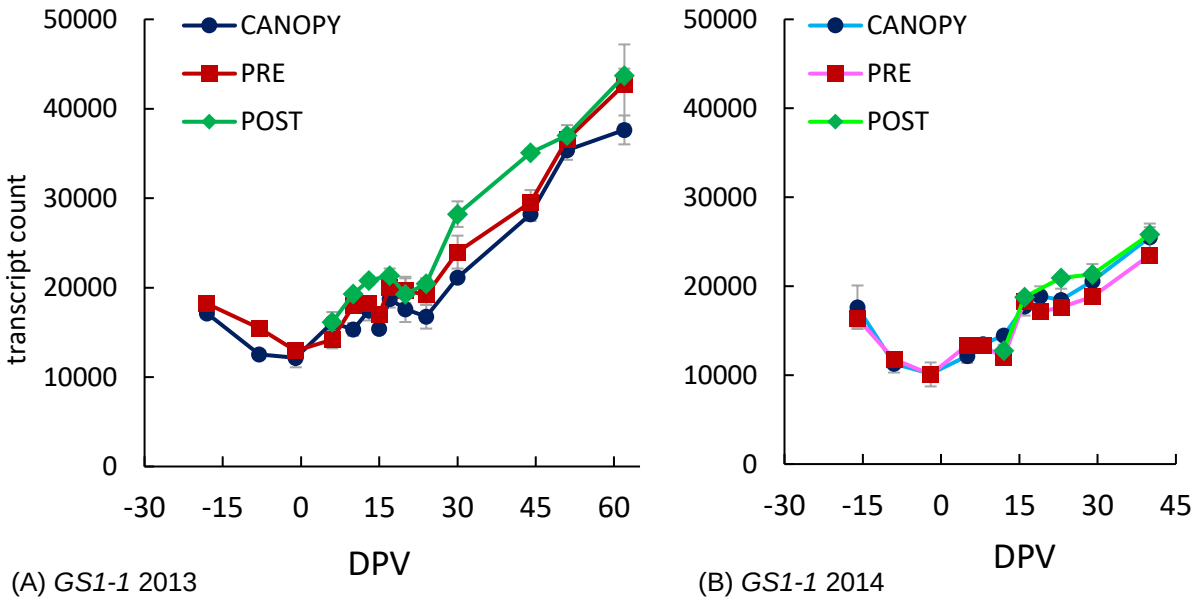


Figure 5.3 ***VvGS1-1*** **nCounter transcript analysis in Sauvignon blanc grapes.**

Transcript counts are shown through berry development in the: (A) 2013 season and (B) 2014 season, comparing transcript abundance in CANOPY control samples to preveraison (PRE) and postveraison (POST) leaf removal treatments. Sampling times are represented with respect to veraison; -18 to 62 days postveraison (DPV) in 2013, and -16 to 40 DPV in 2014. Each data point is the mean ± SEM (n = 3).

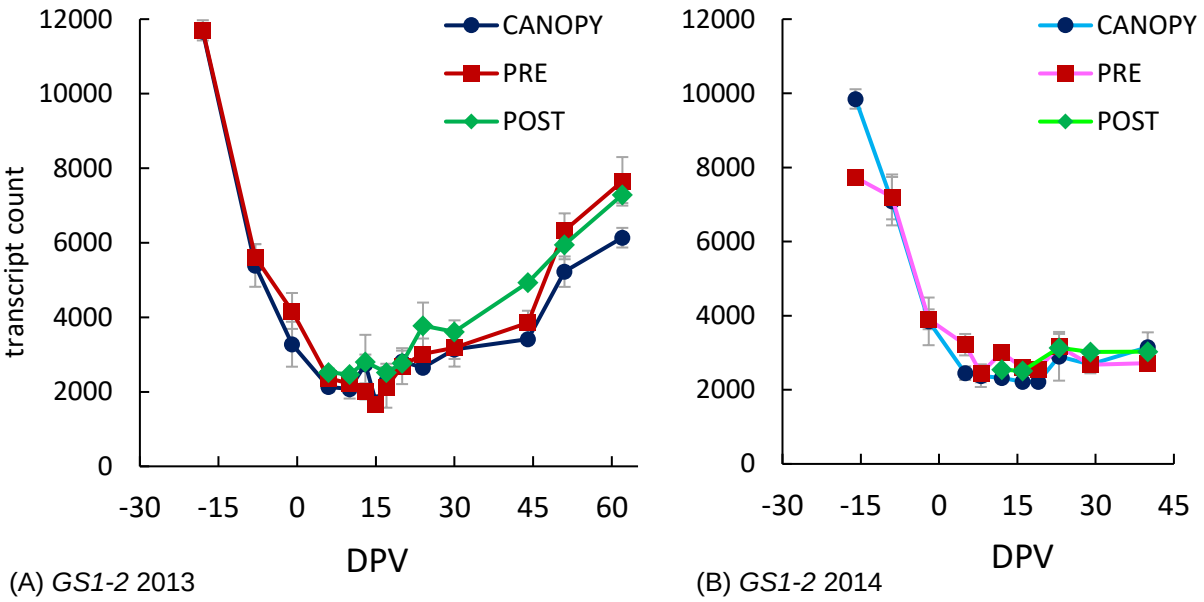


Figure 5.4 ***VvGS1-2*** **nCounter transcript analysis in Sauvignon blanc grapes.**

Transcript counts are shown through berry development in the: (A) 2013 season and (B) 2014 season, comparing transcript abundance in CANOPY control samples to preveraison (PRE) and postveraison (POST) leaf removal treatments. Sampling times are represented with respect to veraison; -18 to 62 days postveraison (DPV) in 2013, and -16 to 40 DPV in 2014. Each data point is the mean ± SEM (n = 3).

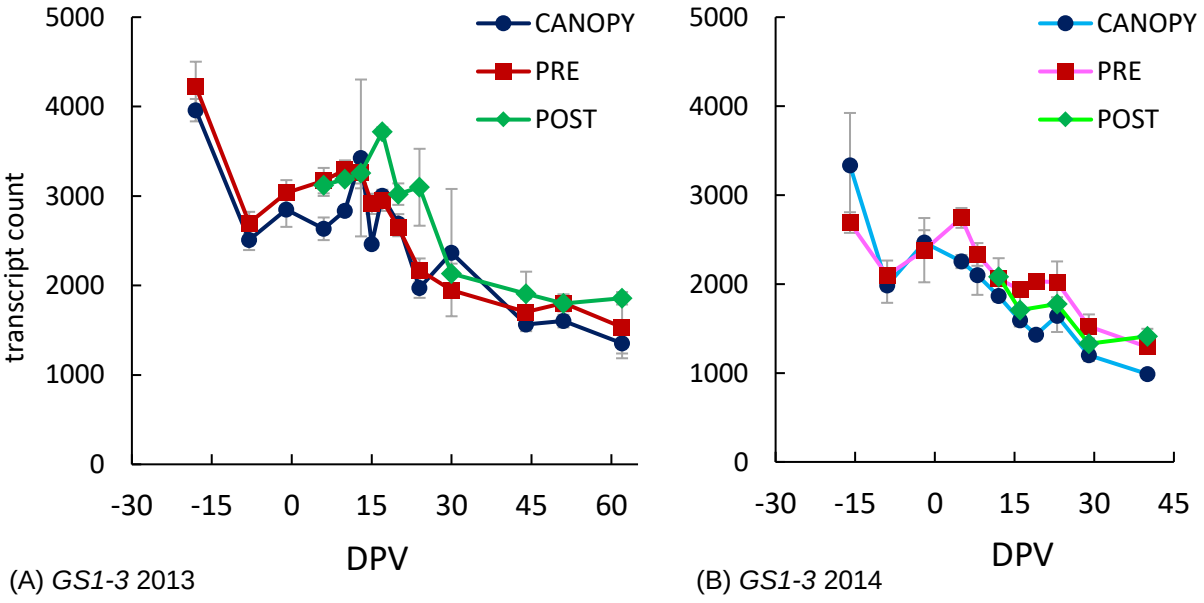


Figure 5.5 ***VvGS1-3*** **nCounter transcript analysis in Sauvignon blanc grapes.**

Transcript counts are shown through berry development in the: (A) 2013 season and (B) 2014 season, comparing transcript abundance in CANOPY control samples to preveraison (PRE) and postveraison (POST) leaf removal treatments. Sampling times are represented with respect to veraison; -18 to 62 days postveraison (DPV) in 2013, and -16 to 40 DPV in 2014. Each data point is the mean ± SEM (n = 3).

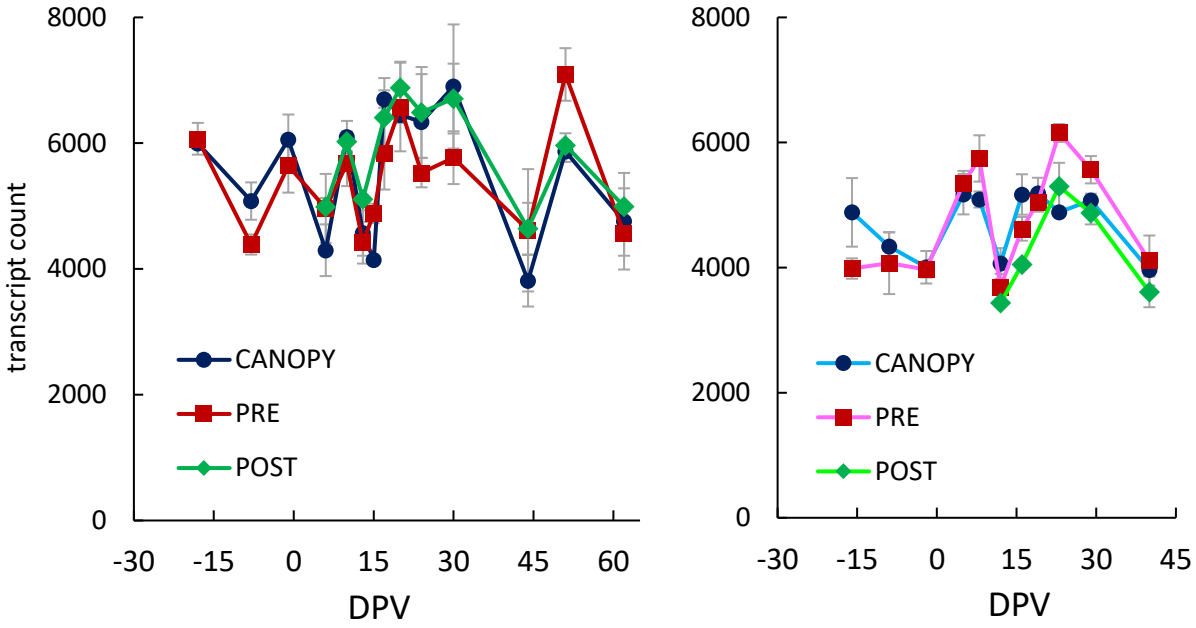
5.4.2 Glutamate synthase (*VvGOGAT*)

As discussed in Chapter 1.6.3, enzyme activity of both classes of GOGAT (Fd- and NADH-) have been demonstrated in grapevine tissues. Previous investigations have revealed two grapevine cDNAs that encode for distinct isoforms of Fd-GOGAT, and Southern analysis indicates that Fd-GOGAT is present as two genes in the grapevine genome (Creasy & Breen 1997; Loulakakis & Roubelakis-Angelakis 1997). Northern analysis demonstrated expression of *Fd-GOGAT* mRNA to be high in grapevine leaves and very low in grape berries. This suggests that Fd-GOGAT is unlikely to play a major role during berry development.

Considerably less is known about expression of *NADH-GOGAT* isoforms in grapevine tissues and especially in berries. As *Fd-GOGAT* isoforms are not highly expressed in grape berries, it is possible that NADH-GOGAT may be the major GOGAT isoform expressed in berries for ammonia assimilation. Therefore, the expression of two transcripts of *NADH-VvGOGAT* (-1 and -2) coding for putative NADH-GOGAT enzyme isoforms were analysed in Sauvignon blanc grape berries through development. We carried out preliminary sequence analysis on these two *NADH-GOGAT* transcripts and show they have a sequence identity of 97% to one another and code for distinct polypeptides. They also have a high homology to known full-length *NADH-GOGAT* cDNAs from other plants that produce active proteins (Goto et al. 1998; Gregerson et al. 1993).

Overall in both seasons, the *NADH-GOGAT-1* transcript is expressed constitutively in berries through development, CANOPY control samples averaging 5505 counts in 2013 and 4712 in 2014 (Figure 5.6). Transcript counts do oscillate through development, but maintain a range of expression in CANOPY control samples between 4054-6902 counts in 2013 and 3967-6153 counts in 2014. Despite these fluctuating profiles of expression, average levels stay generally consistent through development and by the final samplings are statistically equivalent with their preveraison levels. PRE and POST leaf removal treatment samples showed a similar oscillating profiles of *NADH-GOGAT-1* transcript abundance and compared to CANOPY controls, no consistent effects of leaf removal was observed with *NADH-GOGAT-1* expression at any developmental stages measured. The *NADH-GOGAT-2* transcript was detectable at only very low abundance (between 5-35 counts) in both 2013 and 2014, with measurements being inconsistent and at baseline detection thresholds (data not shown).

The relatively high transcript abundance and constitutive expression of *NADH-GOGAT-1* in Sauvignon blanc grape berries suggests that this NADH dependent isoform of GOGAT is likely to play a role in the GS/GOGAT cycle during berry development.



(A) *NADH-GOGAT-1* 2013 (B) *NADH-GOGAT-1* 2014

Figure 5.6 *VvNADH-GOGAT-1* nCounter transcript analysis in Sauvignon blanc grapes.

Transcript counts are shown through berry development in the: (A) 2013 season and (B) 2014 season, comparing transcript abundance in CANOPY control samples to preveraison (PRE) and postveraison (POST) leaf removal treatments. Sampling times are represented with respect to veraison; -18 to 62 days postveraison (DPV) in 2013, and -16 to 40 DPV in 2014. Each data point is the mean ± SEM (n = 3).

5.5 Gene expression analysis of the proline metabolic pathway

In order to understand potential mechanisms for regulation of proline accumulation in Sauvignon blanc grape berries, a number of genes involved in proline metabolism were investigated.

5.5.1 Pyrroline-5-carboxylate synthetase (VvP5CS) and pyrroline-5-carboxylate reductase (*VvP5CR*)

The two-step process of biosynthesis in which glutamate is converted to proline via a GSA/P5C intermediate, is catalysed two successive reactions catalysed by P5CS and P5CR, respectively. *VvP5CS* expression has previously been shown relatively consistent through berry development (Stines et al. 1999) and more recent research reported two additional putative *P5CS* transcripts (uncharacterised isogenes *P5CS1a* and *P5CS1b*) that were shown to upregulated in ripening berries (Rienth et al. 2014). Conversely to *P5CS*, the profile of *VvP5CR* expression in grape berries has so far not been reported.

To determine how the expression of the proline biosynthetic genes may differ during grape development, *P5CS*, putative isogenes *P5CS1a* and *P5CS1b* and *P5CR* transcripts were analysed in Sauvignon blanc berry samples. As both quantitative and qualitative aspects of proline accumulation are affected by basal leaf removal treatments, it opens up the possibility that transcriptional changes of biosynthetic genes regulated by leaf removal may be responsible for variations in proline accumulation.

In both seasons, *P5CS* is highly expressed throughout berry development (Figure 5.7). *P5CS* transcript abundance in control samples increases 1.5 to 2-fold through veraison to peak at 30 DPV and 29 DPV in 2013 and 2014 respectively. From these maximum transcript counts, *P5CS* expression decreases in both years and is slightly lower by the final samplings at 62 DPV and 40 DPV respectively. Nevertheless, the high transcript counts of *P5CS* and accompanying increase in expression postveraison, overlap with the period in berry development when proline accumulation in the berry is increasing significantly. Conversely to other reports in the literature, the putative isogene transcripts of *P5CS1a* and *P5CS1b* were not detected at any stages of berry development measured (transcript counts less than 5, data not shown).

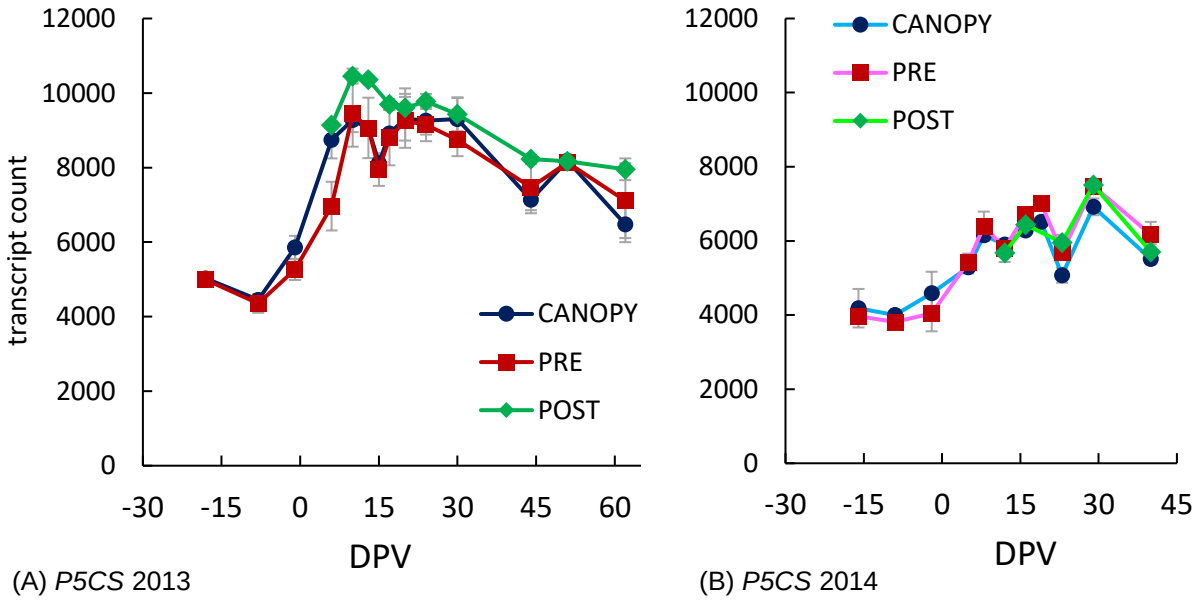


Figure 5.7 *VvP5CS* nCounter transcript analysis in Sauvignon blanc grapes.

Transcript counts are shown through berry development in the: (A) 2013 season and (B) 2014 season, comparing transcript abundance in CANOPY control samples to preveraison (PRE) and postveraison (POST) leaf removal treatments. Sampling times are represented with respect to veraison; -18 to 62 days postveraison (DPV) in 2013, and -16 to 40 DPV in 2014. Each data point is the mean ± SEM (n = 3).

Compared to the *P5CS* proline biosynthetic gene, the transcript abundance of *P5CR* is significantly lower in both seasons at all stages of development (Figure 5.8). *P5CR* transcript counts range between 5 to 10-fold less than *P5CS* at the equivalent developmental time points. Nevertheless, expression of *P5CR* is constitutive and reasonably consistent through development in both seasons, transcript counts maintaining a range of expression in CANOPY control samples between 792-1266 counts in 2013 and 622-935 counts in 2014.

The transcript abundance of *P5CS* and *P5CR* was not affected by the leaf removal treatments (PRE and POST), when compared to the CANOPY control treatments. This observation suggests that there is no differential expression induced by our experimental leaf removal treatments. It is therefore likely that differential expression of these two biosynthetic transcripts is not responsible for quantitative and qualitative changes seen in proline accumulation in the grape berry.

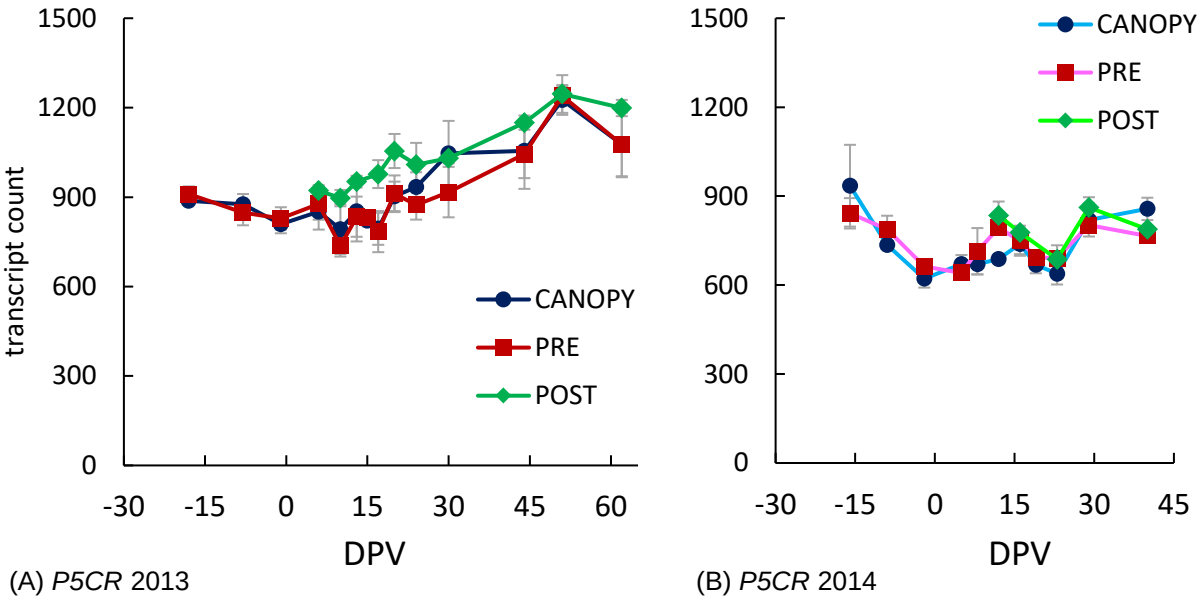


Figure 5.8 *VvP5CR* nCounter transcript analysis in Sauvignon blanc grapes.

Transcript counts are shown through berry development in the: (A) 2013 season and (B) 2014 season, comparing transcript abundance in CANOPY control samples to preveraison (PRE) and postveraison (POST) leaf removal treatments. Sampling times are represented with respect to veraison; -18 to 62 days postveraison (DPV) in 2013, and -16 to 40 DPV in 2014. Each data point is the mean ± SEM (n = 3).

5.5.2 Proline dehydrogenase (*VvPDH*) and pyrroline-5-carboxylate dehydrogenase (*VvP5CDH*)

The first step of proline degradation in plants is mediated by the enzyme PDH which converts proline to P5C. The subsequent action of P5CDH completes the catabolism of proline by catalysing the reaction of P5C back to glutamate. A coordinate and reciprocal regulation of *PDH* and *P5CS* expression has been shown to tightly control proline levels in some plants and this has shown to be particularly important during periods of osmotic stress (Kiyosue et al. 1996; Nakashima et al. 1998; Peng et al. 1996). In grapes, levels of the PDH protein increases during postveraison stages of berry development, which has indicated that the accumulation of proline in grape berries is not due to a decrease in proline degradation (Stines et al. 1999). Although, direct measurements of PDH activity have not been demonstrated in grapes to confirm this hypothesis. Additionally, the expression profiles of *VvPDH* and *VvP5CDH* mRNAs in developing grape berries have yet to be investigated.

In order to examine whether enhanced proline levels in ripening berries could result from changes in expression of proline degradation genes, the abundance of *PDH* and *P5CDH* transcripts were analysed in Sauvignon blanc berry samples. It was also determined if leaf removal treatments had any effect on the expression of *PDH* and *P5CDH* transcripts.

PDH transcript counts are at relatively low levels preveraison, being at their lowest levels around veraison in both seasons (Figure 5.9). *PDH* transcripts increase substantially postveraison in all treatments to be 4-fold higher by 62 DPV in 2013 and 2 to 3-fold elevated at 40 DPV in 2014. During the postveraison ripening stage of development in both seasons, transcripts counts in the PRE and POST leaf removal treatments are consistently greater compared to CANOPY control samples at the same time points. This is an interesting observation as any increase in expression of transcripts involved in proline degradation pathways could potentially impact proline concentrations in the berry (as seen with the leaf removal treatments).

The transcript abundance of *P5CDH* is higher in both seasons, when compared to the transcript counts of *PDH* (Figure 5.10). *P5CDH* transcript counts range between 5 to 10-fold higher than *PDH* counts at the equivalent developmental time points. The relatively high expression of *P5CDH* is constitutive and remarkably consistent through development in both seasons, transcript counts maintaining a range of expression in control samples between 4953-6649 counts in 2013, and 3531-5354 counts in 2014. The transcript abundance of *P5CDH* was not affected by the leaf removal treatments (PRE and POST), when compared to the CANOPY control treatments.

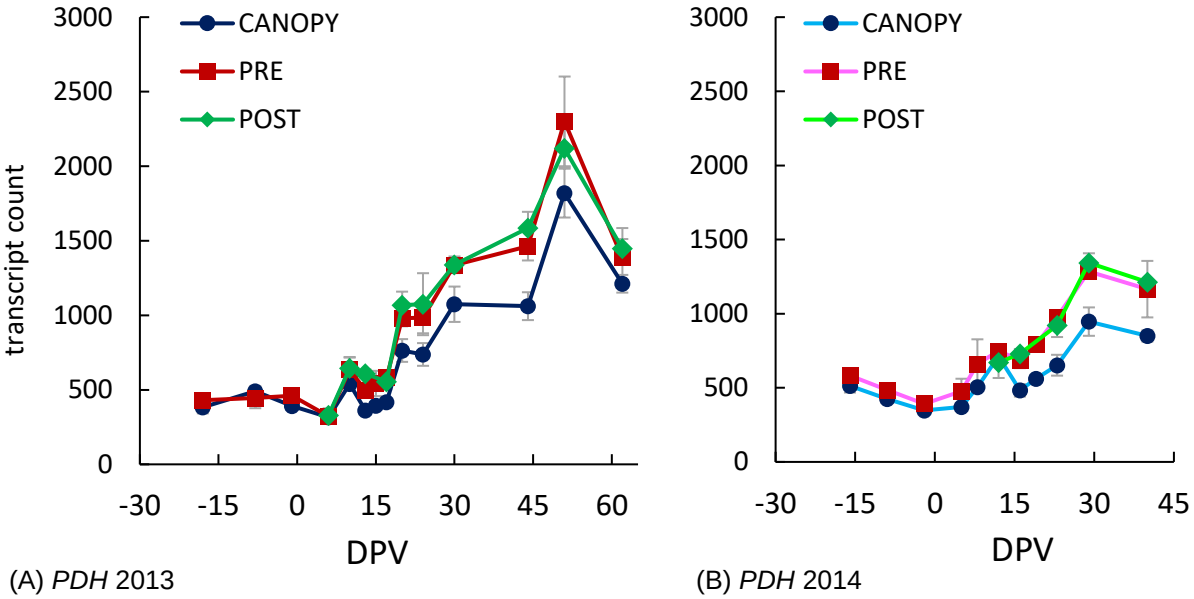


Figure 5.9 ***VvPDH*** **nCounter transcript analysis in Sauvignon blanc grapes.**

Transcript counts are shown through berry development in the: (A) 2013 season and (B) 2014 season, comparing transcript abundance in CANOPY control samples to preveraison (PRE) and postveraison (POST) leaf removal treatments. Sampling times are represented with respect to veraison; -18 to 62 days postveraison (DPV) in 2013, and -16 to 40 DPV in 2014. Each data point is the mean ± SEM (n = 3).

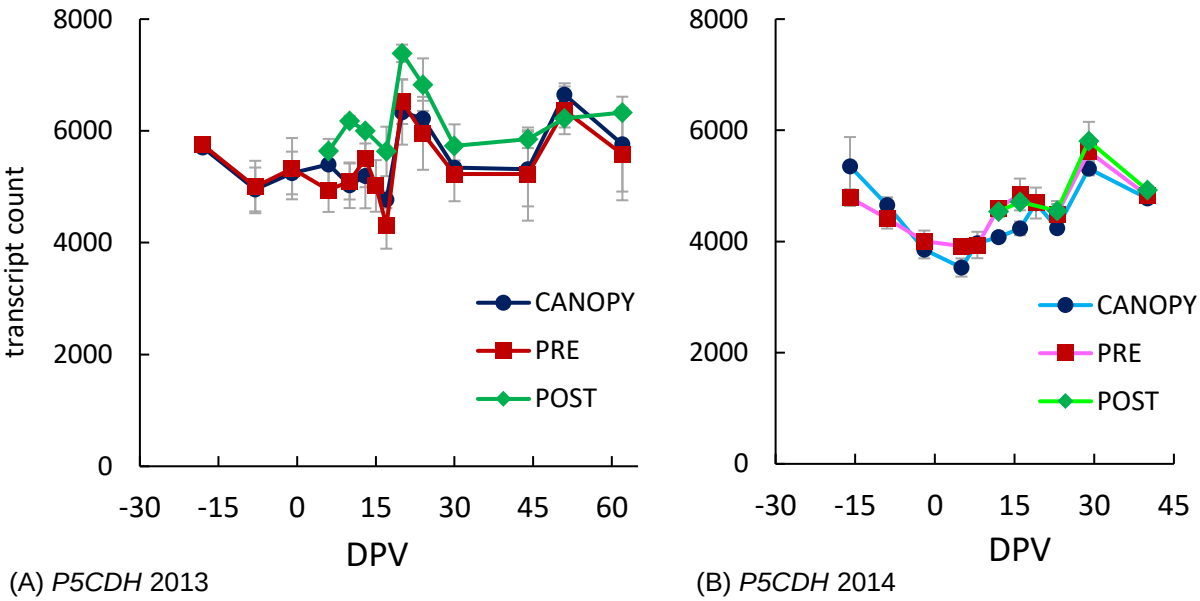


Figure 5.10 ***VvP5CDH*** **nCounter transcript analysis in Sauvignon blanc grapes.**

Transcript counts are shown through berry development in the: (A) 2013 season and (B) 2014 season, comparing transcript abundance in CANOPY control samples to preveraison (PRE) and postveraison (POST) leaf removal treatments. Sampling times are represented with respect to veraison; -18 to 62 days postveraison (DPV) in 2013, and -16 to 40 DPV in 2014. Each data point is the mean ± SEM (n = 3).

5.6 Gene expression analysis of a (potential) alternative proline biosynthetic pathway

To investigate the potential of an alternative biosynthetic pathway to proline from arginine and ornithine precursors, the expression profiles of the genes arginase and ornithine aminotransferase (*OAT*) and the stress-responsive NAC2 (*SNAC2*) gene (a transcription factor and activator of OAT) were analysed throughout development.

5.6.1 Arginase and ornithine aminotransferase (VvOAT)

The patterns of arginine and proline accumulation in ripening grape berries have led to hypotheses that their metabolism may be linked, with the potential for arginine to act as an "secondary" precursor for enhancing proline accumulation. This alternative pathway for proline biosynthesis is through the activity of arginase and OAT via an ornithine (and P5C) intermediate and is not just a model in grapes, but has been postulated for many plants (see Chapter 1.9).Differential compartmentalisation of the substrates and enzymes involved has meant this model has proven controversial. The ability of P5C to directly contribute to the synthesis of proline would require transport out of the mitochondria into the cytosol by an as yet unknown mechanism. But even if P5C cannot be directly utilised for proline biosynthesis, this pathway may still be important in developing grape berries. Any P5C produced through the activity of arginase and OAT could be utilised by P5CDH to "regenerate" glutamate, which can then be diverted back into amino acid pathways, including as a precursor for proline. Both arginase and OAT activity and *VvOAT* mRNA have been detected in grape berries, albeit at low levels (Roubelakis-Angelakis & Kliewer 1981; Stines et al. 1999), while the expression profile of arginase mRNA in grape berries has not been reported.

The roles of arginase and OAT in grape berries have had little attention since these decades old preliminary studies and are far from resolved. Therefore, to further investigate this pathway in grapevine, the expression profiles of arginase and *OAT* mRNA transcripts were examined in developing Sauvignon blanc grape berries and in response to leaf removal treatments.

In 2013, the expression of arginase and *OAT* are remarkably similar, both in their levels of transcript abundance and their profiles of expression through development (Figure 5.11 and Figure 5.12). Both arginase and *OAT* expression are around 2000 transcript counts through veraison, with *OAT* transcript counts initially decreasing through preveraison time points. Expression of both arginase and *OAT* start increasing at 15 DPV, transcript counts increasing to 2.5-fold veraison levels by 62 DPV. As with the 2013 data, the 2014 expression of arginase and *OAT* similarly resemble each other both in their levels of transcripts and their profiles. Transcript counts of each initially decrease to veraison, but then their transcript levels increase postveraison to be 2-fold higher than veraison levels by 40 DPV. The

abundance of *Arginase* and *OAT* transcripts was not affected by the leaf removal treatments (PRE and POST), when compared to the CANOPY control treatments.

High levels of transcript counts of both arginase and *OAT* were measured at all stages of berry development and the transcript abundance of both increased significantly through postveraison time points. The increase in expression of arginase and *OAT* indicates that expression of these two genes is likely to play a role in amino acid biochemical pathways in the grape berry, especially as they accompany developmental stages in the berry when arginine and proline are also accumulating and glutamine concentrations in the berry are being depleted.

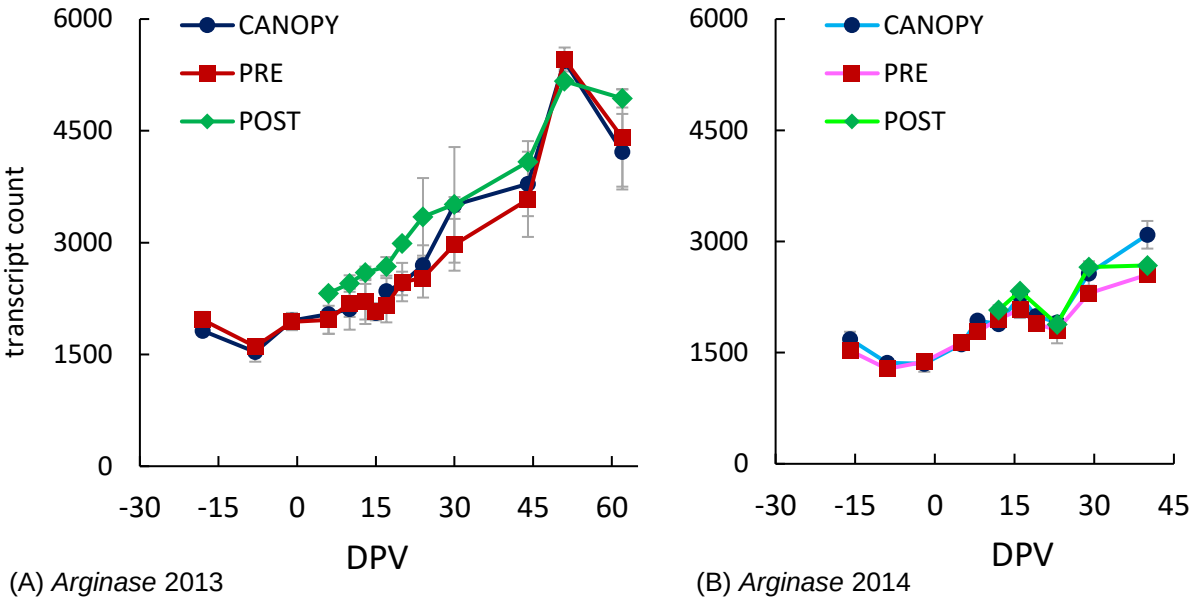


Figure 5.11 *VvArginase* nCounter transcript analysis in Sauvignon blanc grapes.

Transcript counts are shown through berry development in the: (A) 2013 season and (B) 2014 season, comparing transcript abundance in CANOPY control samples to preveraison (PRE) and postveraison (POST) leaf removal treatments. Sampling times are represented with respect to veraison; -18 to 62 days postveraison (DPV) in 2013, and -16 to 40 DPV in 2014. Each data point is the mean ± SEM (n = 3).

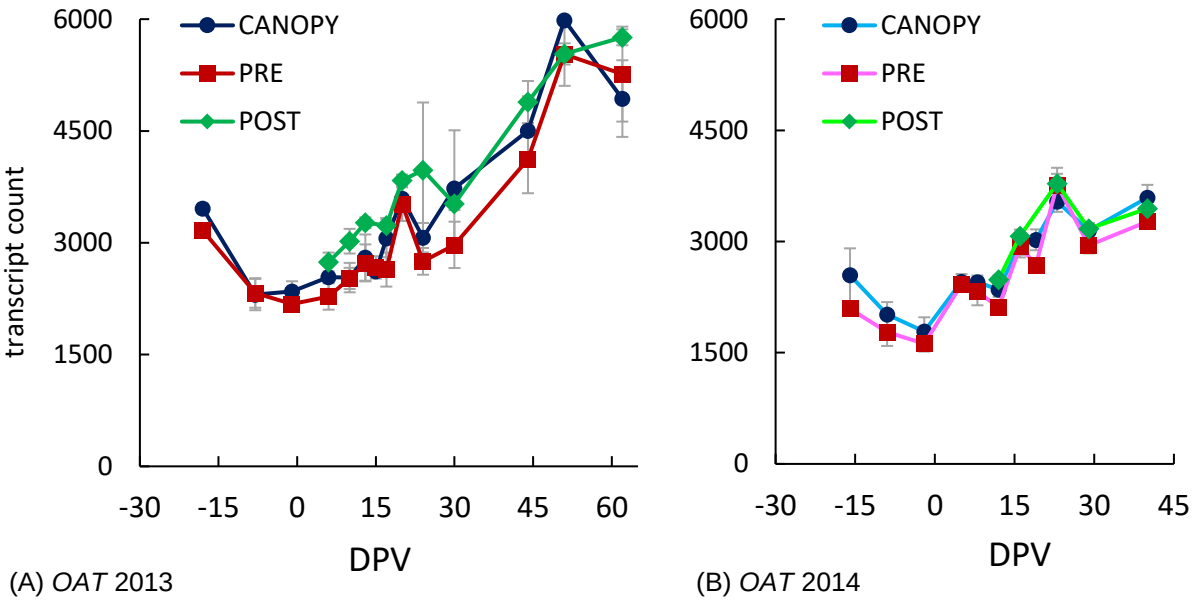


Figure 5.12 *VvOAT* nCounter transcript analysis in Sauvignon blanc grapes.

Transcript counts are shown through berry development in the: (A) 2013 season and (B) 2014 season, comparing transcript abundance in CANOPY control samples to preveraison (PRE) and postveraison (POST) leaf removal treatments. Sampling times are represented with respect to veraison; -18 to 62 days postveraison (DPV) in 2013, and -16 to 40 DPV in 2014. Each data point is the mean ± SEM (n = 3).

5.6.2 Stress-responsive NAC2 (*VvSNAC2*) transcription factor

The NAC family of transcription factors play vital roles in regulating growth and development processes in plants. Within this group of regulatory molecules, SNAC2 is a NAC transcription factor involved in abiotic stress responses. *SNAC2*-overexpressing rice plants for example, exhibit enhanced drought stress responses including salinity and osmotic tolerance (You et al. 2012). One of the up-regulated genes in *SNAC2*-overexpressing rice plants is *OAT* and later characterisation of the *OsOAT* gene and *OAT*-overexpressing rice plants showed significantly increased OAT enzyme activity and enhanced proline levels (Hu et al. 2008). Additionally, the expression of *OsOAT* was directly regulated by SNAC2, potentially through specific binding of SNAC2 to the *OAT* promoter.

There are currently no reports in the literature of the *SNAC2* gene or protein being investigated in grapevine. Therefore, to analyse *SNAC2* expression in Sauvignon blanc grape berries, a predicted *VvSNAC2* (NAC domain-containing protein 2) transcript was identified with significant homology to *SNAC2* transcripts from other plants. The abundance of this *VvSNAC2* transcript was examined in Sauvignon blanc berries throughout development and it was also determined if leaf removal treatments had any effect on its expression.

In both seasons, the transcript abundance of *SNAC2* initially decreases through preveraison time points to veraison, with CANOPY control samples dropping to 218 transcript counts at -1 DPV in 2013 and 247 counts at -2 DPV in 2014 (Figure 5.13). From this low in 2013, *SNAC2* expression starts increasing at 10 DPV and continues increasing substantially postveraison to be 13-fold higher than veraison levels at 62 DPV. Similarly in 2014, *SNAC2* expression starts increasing from 12 DPV to have 6-fold higher transcript counts than veraison levels by the final sampling at 40 DPV. Expression of *SNAC2* showed no response to basal leaf removal in either season with the transcript abundance of the PRE and POST treatments being equivalent to CANOPY controls at all stages of development measured.

The transcript abundance of *SNAC2* is therefore increased at preveraison and postveraison time points in Sauvignon blanc berries, with the least *SNAC2* expression occurring at veraison. This profile of expression is very similar to the expression profile of *OAT*, a gene that is regulated by the SNAC2 transcription factor in other plants. Observing that the postveraison induction in *SNAC2* transcript abundance directly coincides with the postveraison increase in *OAT* expression, strongly indicates that the expression of *SNAC2* may have a role in regulating transcription of *OAT* in grape berries.

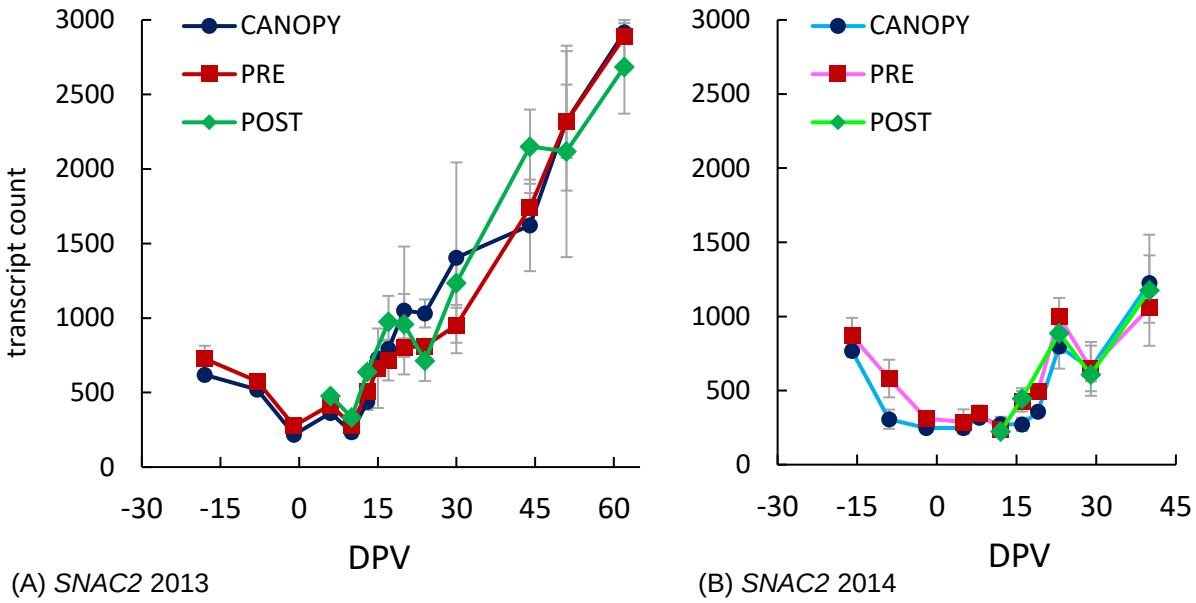


Figure 5.13 *VvSNAC2* nCounter transcript analysis in Sauvignon blanc grapes.

Transcript counts are shown through berry development in the: (A) 2013 season and (B) 2014 season, comparing transcript abundance in CANOPY control samples to preveraison (PRE) and postveraison (POST) leaf removal treatments. Sampling times are represented with respect to veraison; -18 to 62 days postveraison (DPV) in 2013, and -16 to 40 DPV in 2014. Each data point is the mean ± SEM (n = 3).

5.7 Conclusions

The results presented in this chapter quantify the abundance of a range of genes (transcripts) involved in different aspects of metabolism and regulation of the α-ketoglutarate family of amino acids (glutamine, glutamate, arginine and proline).

The most highly expressed *GS1* homolog analysed was the *GS1-1* transcript at all stages of berry development and increasing substantially postveraison. Additionally, the *NADH-GOGAT-1* transcript was highly expressed constitutively in berries through development. It is therefore likely that expression of these two transcripts play a role in the GS/GOGAT cycle during berry development, working in tandem to maintain glutamine and glutamate concentrations, as they are converted to other amino acids which are accumulating in the berry.

The key gene in the proline biosynthetic pathway is *P5CS* and is the first step (and rate-limiting step) in the pathway from its glutamate precursor. *P5CS* is highly expressed throughout berry development and increases significantly through veraison. The increased postveraison expression of *P5CS*, overlap with the period in berry development when proline accumulation in the berry is increasing significantly. *PDH* (the first step in proline catabolism) expression is at relatively low levels preveraison, subsequently increasing substantially through postveraison time points. This increased postveraison expression in the berry is occurring at the same time when proline quite clearly continues to accumulate regardless of the increased expression of *PDH*. The transcript abundance of *PDH* in the PRE and POST leaf removal treatments were consistently greater compared to CANOPY control samples during postveraison time points. Any changes noted in expression of transcripts (with respect to the leaf removal treatments) involved in proline catabolism could potentially impact proline concentrations in the berry. Although not impacted by the leaf removal treatments, the relatively high and constitutive expression of *P5CDH* through development, additionally suggests a significant role of this transcript in both proline metabolism and arginine catabolism in the grape berry.

An alternative biosynthetic pathway leading to production of P5C (and potential proline synthesis), from arginine and ornithine is highly expressed in grape tissue throughout development. Arginase and *OAT* expression increase significantly through development, along with the transcription factor *SNAC2*, which is widely reported to be an activator of OAT. The increase in transcript abundance of arginase, *OAT* and *SNAC2* indicates that expression of this pathway is likely to play a major role in amino acid metabolism in the grape berry.

Chapter 6
Characterisation of an OAT activity assay in Sauvignon blanc grapes

6.1 Introduction

The results obtained from the nCounter transcript analysis demonstrated that the genes responsible for the potential biosynthetic pathway linking arginine to proline metabolism (via ornithine and P5C intermediates), are expressed throughout development in Sauvignon blanc grape berries. In particular, arginase and *OAT* gene expression are upregulated postveraison, overlapping the period of development when both arginine and proline are accumulating in the berry. Expression of the transcription factor *SNAC2*, which has been shown to regulate *OAT* expression in other plants, is also up-regulated postveraison and at the same time as arginase and *OAT* transcripts are increasing in the berry.

These observations suggest that stimulation of this pathway could contribute to support proline accumulation (or at least regeneration of glutamate in the first instance) in Sauvignon blanc grapes. As discussed in Chapters 1 and 5, the production of P5C through this pathway may not be able to be directly diverted back into proline biosynthesis because of compartmentalisation issues; with OAT activity producing P5C in the mitochondria and the proline biosynthetic enzymes being confined to the cytosol. However, the catabolic gene P5CDH was shown to be constitutively expressed with high transcript abundance through Sauvignon blanc berry development and the P5CDH enzyme is known to be located in the mitochondria in other plants. It is therefore probable that at least some of the P5C produced through the activity of arginase and OAT could be utilised by the P5CDH enzyme, forming glutamate. Furthermore, as well as producing P5C, the reaction of ornithine with α-ketoglutarate catalysed by the OAT enzyme, also forms glutamate (see Figure 1.11). This could be a way for the berry to mobilise reserves of nitrogen stored as arginine, as concentrations of glutamine in the berry (and being imported into the berry) are decreasing. The glutamate produced by OAT and P5CDH can re-enter the GS/GOGAT cycle to then be redirected back into other amino acid pathways, including the proline biosynthetic pathway.

Previous studies have shown *OAT* mRNA, OAT protein and enzyme activity all to be at low levels in several grapevine cultivars (not assessed in Sauvignon blanc) with the authors suggesting such low levels may not contribute significantly to amino acid metabolism (Stines et al. 1999). However, our results show a relatively high expression and significant up-regulation in the abundance of *OAT* and *SNAC2* transcripts at postveraison time points in Sauvignon blanc berry samples. The results presented in

Chapter 5 additionally showed that preveraison and postveraison leaf removal had no impact on the abundance of arginase, *OAT* and *SNAC2* transcripts. It therefore appears that a differential expression of these genes is not responsible for any changes in concentrations of the related amino acids as seen in the berry with respect to leaf removal treatments.

Aside from transcriptional regulation of the metabolic genes, another mechanism that could affect quantitative or qualitative aspects of amino acid metabolism is the allosteric regulation and/or relative activity of associated enzymes. Therefore, to further examine the mechanisms of amino acid accumulation, specifically the production of P5C from an ornithine precursor, the OAT protein enzyme activity was investigated in Sauvignon blanc grape berries. It was of interest to identify if OAT enzyme activity could be detected in Sauvignon blanc grapes through development and if any differences in the relative activity of OAT could be detected in response to basal leaf removal treatments.

6.2 The grapevine OAT activity assay

OAT catalyses the reaction of ornithine and α-ketoglutarate to form P5C and glutamate and is a pyridoxal phosphate dependent enzyme located in the mitochondria (Funck et al. 2008; Kim et al. 1994; You et al. 2012). The assay procedure used for analysing OAT activity in Sauvignon blanc berry samples was a variation on the methods reported by Kim et al. (1994) and Funck et al. (2008), and the specific assay conditions are described in detail in Chapter 2.8 The procedure utilises the reaction of P5C (the target enzymatic product) with ninhydrin under hot acidic conditions to form a water-insoluble reddish pigment. After precipitation by centrifugation, the water insoluble pigment is dissolved in ethanol and the absorbance measured at 510 nm (Ab_{510}).

In the present study, OAT enzyme activity was analysed from whole Sauvignon blanc grape extracts through berry development. Assessing the OAT enzyme using an unpurified extract (homogenised berry samples in assay buffer) was chosen as the method of choice for determining endogenous levels of enzyme activity in *in vitro* assays. By assaying extracts with minimal intervention during the extraction procedure, it was hoped to be able to determine relative specific activity differences between control and leaf removal treatment samples.

Other ways of characterising enzyme activity were considered, such as cloning the *OAT* cDNA encoding the individual protein into an expression vector and expressing the cloned protein heterologously. While this method can facilitate purification and biochemical characterisation of individual enzymes, expressing recombinant proteins in a prokaryotic host can be accompanied with its own set of issues to troubleshoot (Jonasson et al. 2002). This method would also not allow a comparison of relative activity of enzymes with respect to any experimental treatments. It was therefore decided the best approach was to assay OAT activity using fresh, unpurified extracts from whole Sauvignon blanc berry samples. The experiments presented below explore the troubleshooting, optimisation and final results of these OAT activity assays.

6.3 Optimisation of the OAT activity assay

Enzyme activity reactions were set-up as described in Chapter 2.8. Reaction controls were included in which the ornithine substrate and/or extract were omitted from the reaction mixture. This gave the background levels of Ab_{510} and provided a measure of endogenous P5C levels in the absence of external substrate and/or extract addition. As mentioned above, the target reaction product of P5C reacts with ninhydrin under hot acidic conditions to form a water-insoluble reddish precipitate, which is subsequently centrifuged to pellet and then is dissolved in ethanol. An example of the water-insoluble red precipitate produced in the activity assay is shown in Figure 6.1, which demonstrates a colourless reaction control tube (without enzyme extract, see Chapter 6.3.1 below) compared to a tube containing

extract and substrate producing a deep red precipitate. OAT activity measurements were conducted using the FLUOstar omega microplate reader which allowed assays to be performed in smaller volumes and with increased throughput.

Figure 6.1 Reaction of P5C with ninhydrin in OAT activity assays.

The reaction of P5C with ninhydrin under hot acidic conditions, forms a water-insoluble red precipitate that can be dissolved in ethanol and quantified by measuring the absorbance at 510 nm. (A) The reaction controls (RC) contain no enzyme extract and demonstrate no colour production and (B) reaction assays containing enzyme extract and substrate produce a deep red precipitate.

The appropriate reaction control absorbance measurements were subtracted from each assay before any subsequent calculations were performed. Activity was determined by subtracting the Ab_{510} measurements of reactions without substrate, from Ab_{510} measurements of reactions with external substrate addition. Activity was expressed as change in absorbance (over the incubation time of the assay), measured at 510 nm ($\mathbf{\Delta}Ab_{510}$). The protein concentration of extracts was determined by a Bradford assay and used to normalise results to a measure of specific activity ($\mathbf{\Delta}Ab_{510}$/ng protein). Specific activity amounts were further converted to arbitrary fold-change units in which CANOPY control samples were set to 1, with other samples/treatments given as relative.

6.3.1 Reaction controls (RC)

Appropriate reaction controls (RC) were included in each assay, to provide background Ab_{510} measurements to be subtracted from the sample reactions before any subsequent calculations were performed. Reaction controls were processed identically to the sample assay replicates, but had the enzyme extracts omitted from the reaction which was instead replaced with the appropriate volume of assay buffer.

Table 6.1 Reaction controls (RC) provide background measurements for OAT activity assays.

Reaction control	+ / - assay buffer	+ / - α-ketoglutarate	+ / - ornithine (substrate)	Time (min)	Average Ab_{510}*
RC 1	+	-	-	0	0.1195
RC 1	+	-	-	20	0.1096
RC 1	+	-	-	40	0.1122
RC 2	+	+	-	0	0.1174
RC 2	+	+	-	20	0.1160
RC 2	+	+	-	40	0.1184
RC 3	+	+	+	0	0.1567
RC 3	+	+	+	20	0.1502
RC 3	+	+	+	40	0.1532
Ethanol	n/a	n/a	n/a	n/a	0.1156

*Values are means (n = 3).

No increase in Ab_{510} was detected when both enzyme extract and ornithine (substrate) were excluded as shown with RC1 and RC2 (Table 6.1). For comparison, pure ethanol (which is used in the last step of the protocol for extracting the reaction product, P5C, gave equivalent Ab_{510} measurements as RC1 and RC2. RC3 which contained the ornithine substrate in addition to assay buffer and α-ketoglutarate gave a small increase in Ab_{510} compared to RC1 (assay buffer alone). This was an expected result as ornithine is known to give some colour development with detection by ninhydrin in this assay (Kim et al. 1994). However, this assay is optimised with respect to a high acidity (0.6 N using perchloric acid) and this reduces the Ab_{510} of the coloured product produced with ornithine, with the pigment produced by P5C retaining an intense colour in comparison (Kim et al. 1994). Nevertheless, RC2 and RC3 reaction controls were included in every enzyme assay and the Ab_{510} measurements were subtracted from the appropriate sample reactions (i.e. +/- ornithine substrate). This removed background absorbance before any subsequent calculations were performed to calculate $\mathbf{\Delta}Ab_{510}$ and specific activities ($\mathbf{\Delta}Ab_{510}$/ng protein).

6.3.2 Effect of centrifugation on OAT activity

After grinding berry samples in assay buffer, a centrifugation step is included in the protocol to pellet and remove cellular material and debris (see Chapter 2.8). The resulting supernatant was considered the OAT enzyme extract and it was this extract that was used for determining the total protein content and subsequent activity assays. To determine the optimal level of centrifugation in order to remove most of the cellular debris while maintaining the majority of activity, OAT enzyme assays were conducted on extracts after changing the centrifugation speeds and times. All extracts were prepared from freshly picked Sauvignon blanc berry samples.

Table 6.2 Effect of centrifugation on OAT activity assays.

Centrifugation step of protocol	+ / - substrate	Average Ab_{510}* 20 min incubation (RC corrected)	ΔAb_{510}	Specific activity (ΔAb_{510}/ng protein)	Relative specific activity
Uncentrifuged	-	0.2415			
Uncentrifuged	+	0.2854	0.0439	3.73×10^{-7}	1
0.1 rcf, 30 sec	-	0.2177			
0.1 rcf, 30 sec	+	0.2492	0.0315	3.27×10^{-7}	0.876
1.5 rcf, 1 min	-	0.0638			
1.5 rcf, 1 min	+	0.0703	0.0065	7.28×10^{-8}	0.195
18 rcf, 15 min	-	0.0553			
18 rcf, 15 min	+	0.0544	-0.0009	n/a	n/a

*Average Ab_{510} values are means (n=3).

Increasing centrifugation speed and time of sample extracts had a significant effect on measured Ab_{510} (Table 6.2). The crude extract with the centrifugation step omitted showed the highest change in absorbance measured at 510 nm (Ab_{510}). While this result was not unexpected, this crude extract was also the hardest to work with. Because none of the cellular material had been removed from the extract, it was difficult to pipette with accuracy (and a consistency between samples) and for this reason gave inconsistent readings when assaying replicates from the same and different samples. The extract that showed the next highest change in Ab_{510} was the extract that had been centrifuged at 0.1 rcf for 30 sec. This centrifugation regime removed most the cellular debris while maintaining approximately 72% change in Ab_{510} and improved accuracy to allow more consistent measurements between replicates. Extracts that had been centrifuged at 1.5 rcf for 1 min showed considerably less measurable change in Ab_{510}, while the ability of the extracts to give an increase in Ab_{510} disappeared after the samples were centrifuged at 18 rcf for 15 min.

When the total protein content of extracts was taken into account and changes in Ab_{510} converted into specific activity (ΔAb_{510}/ng protein), results showed smaller differences between the (uncentrifuged) crude extracts and the extracts centrifuged at 0.1 rcf for 30 sec. Compared to the uncentrifuged extract, the sample extracts centrifuged at 0.1 rcf (30 s) showed 87% of the specific activity and demonstrated a greater accuracy and consistency between replicates. Whereas the extracts that had been centrifuged at 1.5 rcf (1 min) showed a 5-fold less (19.5%) specific activity compared to the uncentrifuged extract and extracts centrifuged at 18 rcf (15 min) had no specific activity.

It was therefore decided that the optimal centrifugation regime of extracts for all subsequent assays was 0.1 rcf for 30 s. This enabled the extracts to maintain a relatively high level of specific activity (as measured by ΔAb_{510}/ng protein), but also increased accuracy to allow more consistent measurements and better standardisation between replicates and samples.

6.3.3 Effect of extract volume, incubation time and heat inactivation on OAT activity

To further optimise OAT activity assays, the extract volume, assay incubation times and heat inactivation of the extract were studied to determine their effect on Ab_{510} measurements. Experiments were performed in which 100 µL extracts and 200 µL extracts were incubated (both with and without ornithine substrate) for 0 min, 20 min and 40 min. All assays were set-up identically, but then the 0 min assays had perchloric acid added straight after, thereby immediately terminating the reaction. Also included in the 20 min and 40 min time points was the addition of a reaction that contained 200 µL extract that had been heat inactivated by boiling at 100°C for 5 min in a water bath. This heat inactivated reaction was otherwise treated identically to and incubated with the other sample reactions. All extracts were prepared from freshly picked Sauvignon blanc berry samples.

The doubling of the amount of extract from 100 µL to 200 µL should have the effect of doubling the average Ab_{510}. This would show that by doubling the extract volume, the amount of P5C that is detected has also doubled. As can be seen in Table 6.3, after RC correction with the corresponding RC controls, the ratio of Ab_{510} at 0 min between 200 µL extract/100 µL extract was 1.86 and 1.97, without and with ornithine substrate respectively. This trend continues with reactions after 20 min and 40 min incubations. This result demonstrates that doubling the amount of extract in the assay, doubles the Ab_{510}. Therefore in our assay, within the confines of the extract volumes tested, the extract volume is proportional to the Ab_{510} and amount of detectable P5C.

Table 6.3 Effect of extract volume, incubation time and heat inactivation on OAT assays.

Reaction	+ / - substrate	Time (min)	Average Ab_{510}* (RC corrected)	Ratio of Ab_{510} (200 uL extract/100 uL)	ΔAb_{510}	Ratio of ΔAb_{510} (200 uL extract/100 uL)	Specific activity (ΔAb_{510}/ng protein)	Relative specific activity
100 µL extract	-	0	0.0878					
100 µL extract	+	0	0.0826					
200 µL extract	-	0	0.1629	1.86				
200 µL extract	+	0	0.1635	1.97				
100 µL extract	-	20	0.1067					
100 µL extract	+	20	0.1204		0.0137		3.66×10^{-7}	1
200 µL extract	-	20	0.2147	2.01				
200 µL extract	+	20	0.2448	2.03	0.0301	2.17	3.99×10^{-7}	1.09
200 µL extract (B)	+	20	0.2035	n/a				
100 µL extract	-	40	0.1160					
100 µL extract	+	40	0.1266		0.0106		2.82×10^{-7}	1
200 µL extract	-	40	0.2393	2.06				
200 µL extract	+	40	0.2615	2.06	0.0222	2.09	2.96×10^{-7}	1.05
200 µL extract (B)	+	40	0.2059	n/a				

*Average Ab510 values are means (n=3). (B) Indicates heat inactivation (boiling) of the extract prior to set-up of activity assay.

After 20 min incubation, the activity ($\mathbf{\Delta}Ab_{510}$) of reactions was determined by subtracting the Ab_{510} measurements of reactions without substrate, from Ab_{510} measurements of reactions with ornithine addition. Increasing the extract volume from 100 µL to 200 µL doubles the $\mathbf{\Delta}Ab_{510}$ with the ratio of $\mathbf{\Delta}Ab_{510}$ ($\mathbf{\Delta}Ab_{510}$ 200 µL extract/$\mathbf{\Delta}Ab_{510}$ 100 µL extract) being 2.17. This result confirms that the activity of the enzyme is relative to the total protein present in the extract. An increase of extract volume from 100 µL to 200 µL doubles the total protein present in the assay. The amount of enzyme present in the extracts is therefore also doubled, and this is reflected in our assays with a doubling of activity ($\mathbf{\Delta}Ab_{510}$). Once the protein concentration of extracts were determined and used to normalise results to a measure of specific activity ($\mathbf{\Delta}Ab_{510}$/ng protein), the relative specific activity comparing 100 µL extract with 200 µL extract were 1 and 1.09 respectively. This confirmed the validity of the results obtained, as the relative specific activity comparing extract volumes was equivalent when protein content was taken into account.

After 40 min incubation, the main differences compared to the 20 min results were in the $\mathbf{\Delta}Ab_{510}$ measurements and therefore the specific activity results. These activity measurements were decreased at 40 min, even though the average Ab_{510} measurements were slightly higher than the 20 min measurements. A number of causes could account for these observations and could include changes to OAT enzyme stability and enzyme kinetics, stability of the reaction product (P5C) over time and metabolism of P5C by other enzymes.

Heat inactivation through the boiling of enzyme extracts before addition to OAT activity assays, destroyed the ability of the extracts to give an increase in Ab_{510} after 20 min and 40 min incubations, presumably by denaturation of the enzyme protein. The effects of the high temperature treatment on extracts included in activity assays are quite complex. The Ab_{510} in the heat-inactivated (B) 200 µL extract was approximately 0.04 higher than the Ab_{510} in the 200 µL (+ ornithine) assay that was immediately terminated at 0 min. This indicates a higher baseline level of detectable P5C after heat inactivation and subsequent incubation before termination of the assay reactions. These changes in baseline Ab_{510} are most likely due to the heat treatment breaking open cells walls (and/or cell organelles) and releasing their contents. Heating therefore results in changes to the extract composition and subsequent incubation could alter the amount of detected P5C (P5C is spontaneously formed from and is in equilibrium with GSA). Nevertheless, as observed at 20 min and 40 min incubation time points, reactions with heat treated extracts produced no increase in Ab_{510} and show that measured activity ($\mathbf{\Delta}Ab_{510}$) has to be coming from the (non-denatured) sample extracts.

6.3.4 Effect of sample freezing on OAT activity

To explore the feasibility of using frozen berry samples for use in OAT activity assays, previously frozen samples were used to prepare enzyme extracts. Reaction assays were set-up in an identical manner to extracts from freshly picked samples and the ability of the (frozen sample) extracts to give an increase in Ab_{510} was determined.

As seen in Table 6.4, after RC correction, the average Ab_{510} at 0 min were considerably higher, which indicated increased baseline detection of P5C with extracts prepared from frozen samples. Also, the ratio of Ab_{510} at 0 min between 200 µL extract/100 µL extract was 1.69 and 1.72 (without and with ornithine substrate) respectively. The expected ratio of (approximately) 2 was therefore not observed in these frozen extracts. After 20 min incubation, the frozen extracts had average Ab_{510} measurements equivalent to the baseline 0 min readings. Additional boiling (as per Chapter 6.3.3) of the extracts gave no differences to the average Ab_{510} measurements. The ratio of Ab_{510} between 200 µL extract/100 µL extract at 20 min was again less than the expected 2.

Table 6.4 Effect of sample freezing on OAT activity assays.

Reaction	+ / - substrate	Time (min)	Average Ab_{510}* (RC corrected)	Ratio of Ab_{510} (200 uL extract/100 uL)	ΔAb_{510}
100 µL extract (F)	-	0	0.1590		
100 µL extract (F)	+	0	0.1604		
200 µL extract (F)	-	0	0.2687	1.69	
200 µL extract (F)	+	0	0.2766	1.72	
100 µL extract (F)	-	20	0.1514		
100 µL extract (F)	+	20	0.1485		-0.0029
100 µL extract (F & B)	+	20	0.1525		
200 µL extract (F)	-	20	0.2633	1.73	
200 µL extract (F)	+	20	0.2502	1.68	-0.0132
200 µL extract (F & B)	+	20	0.2576	1.69	

*Average Ab_{510} values are means (n=3). (F) Indicates extract was prepared from frozen samples.
(B) Indicates additional heat inactivation (boiling) of the extract prior to set-up of activity assay.

As mentioned above, the results from frozen samples showed higher baseline Ab_{510} measurements at 0 min. The frozen berry samples used had been first ground to a fine powder as per Chapter 2.4, from which a subsample of this frozen grape powder was used to prepare the extracts for activity assays. Therefore, the higher baseline readings are most likely due to the greater maceration of the tissue (by grinding the berry samples) and additional cellular damage from the effect of freezing and thawing. However, aside from these observations, our results show that freezing the samples prior to extract preparation destroys the ability of the extracts to give an increase in Ab_{510}, presumably through denaturation of the enzyme protein, as additional heat inactivation of extracts gave no measurable differences to freezing alone.

6.4 The effect of basal leaf removal on berry OAT activity

To determine if the relative activity of OAT in Sauvignon blanc berries is affected by basal leaf removal treatments, activity assays were conducted at five time points through berry development. The sampled and assayed time points were preveraison (at -6 DPV), postveraison/mid-ripening (at 15, 22 and 36 DPV) and late ripening (50 DPV). CANOPY control (maintained leaf canopy) and PRE (preveraison basal leaf removal) treatment samples from each replicate were assayed together and used to generate specific activity results and a measure of relative specific activity. The result at each assayed time point is therefore the mean of three replicates. The only deviation from this was at the (preveraison) -6 DPV time point, in which the results are from two replicates. This was due to some technical issues with the FLUOstar microplate reader while assaying the third replicate (that were subsequently rectified). All assays were performed on extracts prepared from freshly picked Sauvignon blanc grape berries sampled from the vineyard treatments. As mentioned above, RC2 and RC3 controls were included in every enzyme assay. The RC Ab_{510} measurements were subtracted from the appropriate sample reactions (i.e. +/- ornithine substrate) to remove background absorbance before any subsequent calculations were performed to calculate $\mathbf{\Delta}Ab_{510}$ and specific activities ($\mathbf{\Delta}Ab_{510}$/ng protein).

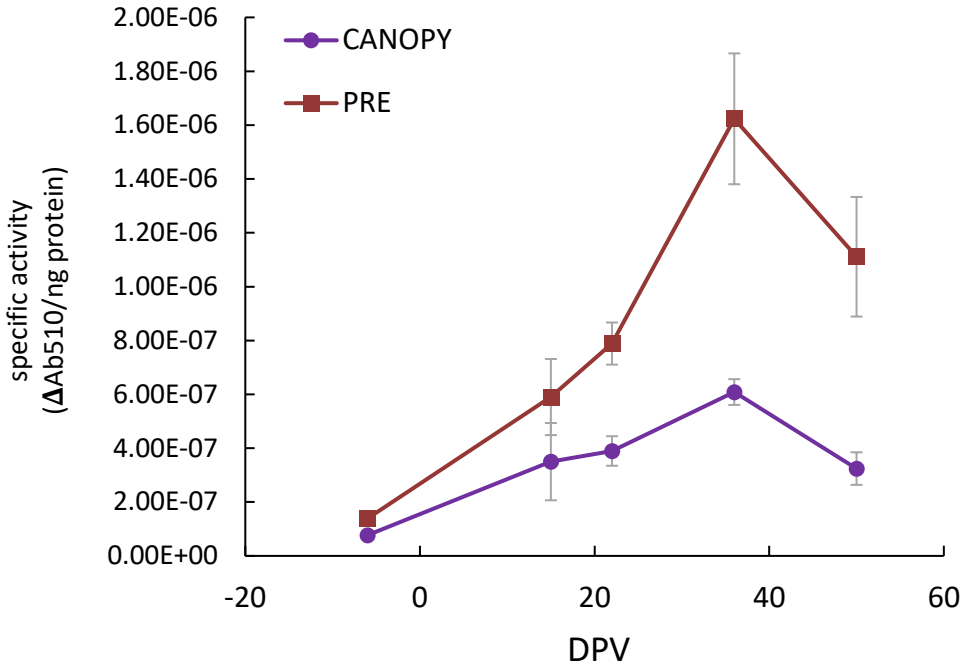


Figure 6.2 Effect of basal leaf removal on specific activity of OAT in Sauvignon blanc grapes.

Specific activity is shown through berry development. Specific activity is calculated as ΔAb_{510} normalised to the protein concentration of extracts (ΔAb_{510}/ng protein) and compare CANOPY control samples to preveraison (PRE) leaf removal treatments. Sampling times are represented with respect to veraison (days postveraison, DPV). Each data point is the mean ± SEM (n = 2 at -6 DPV, n = 3 at other data points).

The results comparing OAT specific activities (ΔAb_{510}/ng protein) in CANOPY control (maintained leaf canopy) and preveraison leaf removal (PRE) treatments are shown in Figure 6.2. Specific activity increases through development in both the CANOPY control and PRE samples from the first sampling time point at -6 DPV, through to peak at 36 DPV. OAT specific activity is then decreased from this peak at the final sampling at 57 DPV in control and PRE treatments. Specific activity in CANOPY control samples are increased 8-fold from -6 DPV to 36 DPV, before decreasing to be 4-fold -6 DPV levels by 52 DPV. The PRE treatment samples increase 12-fold from -6 DPV to 36 DPV, and by the final sampling at 52 DPV are slightly decreased to 8-fold -6 DPV levels. Therefore, compared to CANOPY control samples, OAT specific activity is increased in preveraison leaf removal (PRE) samples at all measured time points, from 14 DPV onwards, through berry development.

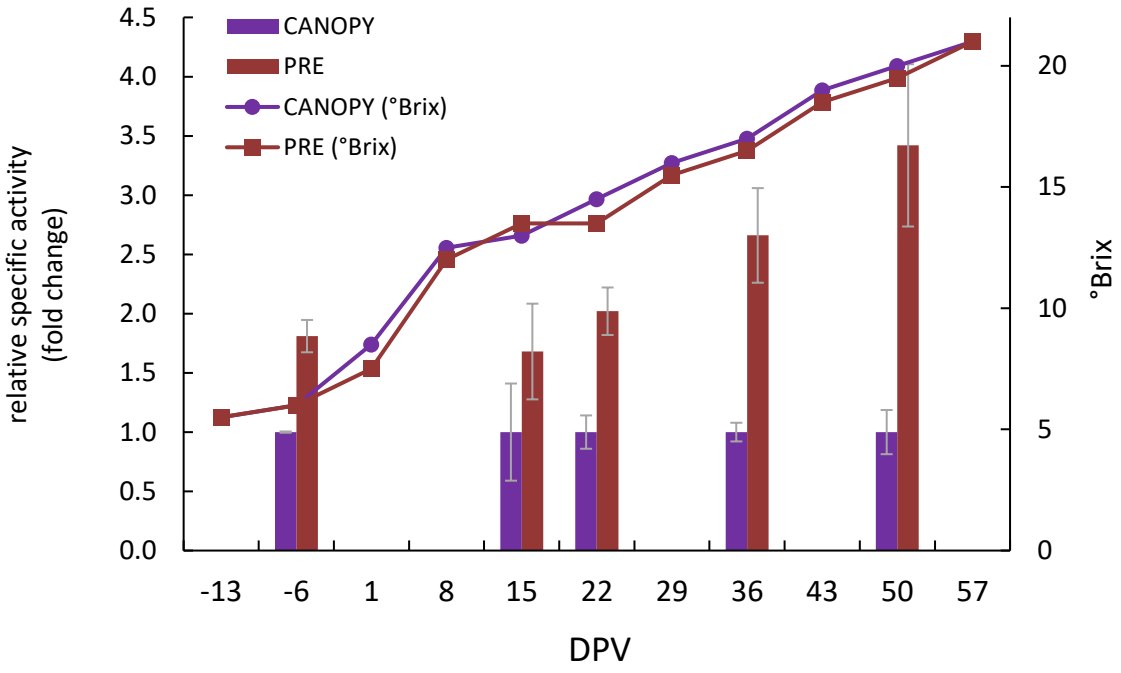


Figure 6.3 Effect of basal leaf removal on relative specific activity of OAT and TSS accumulation in Sauvignon blanc grapes.

Relative specific activity is shown through berry development. The results are shown as fold change comparing CANOPY control samples to preveraison (PRE) leaf removal treatments. Fold changes were determined separately at each time point, with the CANOPY control set to 1 and the PRE samples calculated relative (to the specific activity of the CANOPY control). Sampling times are represented with respect to veraison (days postveraison, DPV). Each data point is the mean ± SEM (n = 2 at -6 DPV, n = 3 at other data points). TSS accumulation (°Brix) in Sauvignon blanc grapes is also shown to illustrate berry development at sampling time points. Each date point is the mean (n = 3).

When the results are converted to relative specific activity it shows the PRE treatment has higher relative specific activity than CANOPY control samples at all measured time points (Figure 6.3). The difference in relative specific activity between CANOPY control and PRE treatment samples, is higher at postveraison/late ripening time points (2.7-fold at 36 DPV and 3.4-fold at 52 DPV).

6.5 Conclusions

The results presented in this chapter investigated OAT enzyme activity in Sauvignon blanc berry extracts through development. The basic biochemical characteristics of the OAT activity assay were tested and optimised in *in vitro* assays using unpurified berry extracts with minimal intervention during the extraction procedure. Reaction controls were included in every activity assay to provide background absorbance (Ab_{510}) measurements. RC measurements were subtracted from the appropriate sample assay reactions before any subsequent calculations were performed. Biochemical characterisation of the OAT activity assays examined the effect of centrifugation (of berry extracts), extract volume, incubation time, sample freezing and heat inactivation of the extract. These conditions were optimised before performing activity assays on the experimental treatment samples.

OAT enzyme assays performed on treatment samples indicate that OAT specific activity (as measured by $\mathbf{\Delta}Ab_{510}$/ng protein) increases through Sauvignon blanc berry development and ripening. Additionally, the maintenance of a leaf canopy appears to be important in regulating OAT activity, as relative specific activity is increased in preveraison leaf removal (PRE treatment) samples at all measured time points through berry development, compared to CANOPY control (maintained leaf canopy) samples.

These results demonstrate that production of P5C through the activity of OAT is likely to play a role in amino acid metabolism in Sauvignon blanc grapes during berry development. This potentially provides a mechanism for the berry to mobilise reserved stores of nitrogen as arginine, back into the GS/GOGAT cycle, ultimately for redistribution into other metabolic pathways.

Chapter 7
Discussion

7.1 Berry phenology and bunch microclimate

7.1.1 The effects of basal leaf removal on berry phenology

In our current study, the berry phenology parameters of accumulation of total soluble solids (°Brix) and berry weight were determined throughout each season. This provided an indication of berry development and maturity with respect to experimental leaf removal treatments in that season. Basal leaf removal from around the grape bunches had no significant effect on the berry phenology parameters measured (TSS and berry weight). Specifically, the accumulation of TSS (°Brix), which is an important measure of berry development, showed no significant differences between the CANOPY control (canopy maintained) treatment and the preveraison (PRE) and postveraison (POST) leaf removal treatments. But as highlighted in Chapter 3.3.1, the result in 2014 indicates that there is the potential for basal leaf removal to have some small effects on TSS accumulation. This result was observed only in the 2014 season, berries from PRE and POST treatments having slightly lower °Brix measurements at postveraison time points. Overall, our results suggest that the essential photosynthesis (carbohydrate production) for berry development was not significantly impacted by basal leaf removal around the fruiting zone/grape bunches. It also suggests that, at least for TSS accumulation and berry weight, that other parts of the canopy (i.e. younger apical leaves and laterals) can "compensate" for the loss of a local (proximal) source of leaves and assimilates.

Previous studies have shown that TSS accumulation in Sauvignon blanc grapes has been found to be relatively unaffected by basal leaf removal (Gregan et al. 2012; Liu et al. 2015; Mosetti et al. 2016; Sivilotti et al. 2017). The effect of early season (before flowering) basal leaf removal has also been investigated in Sauvignon blanc grapes and was shown not to influence fruit phenology (TSS, titratable acidity and pH) parameters at harvest (Sivilotti et al. 2017). Nevertheless, the potential for basal leaf removal to have small effects on TSS accumulation has previously been noted in research that had similar experimental leaf removal treatments to the study presented here. In one year out of a three year study, leaf removal was shown to influence the berries ability to accumulate soluble solids postveraison, slightly reducing °Brix levels in Sauvignon blanc grapes (Gregan & Jordan 2016). The small change in accumulated TSS was not correlated with measured changes in other metabolites. No effect of leaf removal on TSS was reported in the other two years of that study. Research performed using other grape varieties have also shown that basal leaf removal, in general has minimal effects on TSS accumulation (Bavaresco et al. 2008; Conde et al. 2007; Downey et al. 2006, 2004; Poni et al. 2018; Reynolds 2010). TSS accumulation in developing berries relies on the photosynthetic ability and carbon

fixation in mature leaves, and the subsequent allocation and transport of assimilates into the grapes (Conde et al. 2007). In addition, modification of the leaf source (or alteration of sink organs) can potentially alter this balance and can lead to changes in assimilate transport and partitioning. An important point to note is that this current study (and the studies discussed above) are with respect to basal leaf removal on already nutritionally unstressed (balanced) vines. The assumption for basal leaf removal, is that total canopy photosynthesis can be compensated for by other parts of the canopy (further discussed in Chapter 7.3). Although, this may not hold true if leaf removal is applied to already stressed and compromised vines, or the leaf removal is excessive and considerably alters the light exposure and bunch temperatures (Haselgrove et al. 2000; Price 1995; Reynolds 2010).

This key initial result was an important finding for validating the experimental treatments, as it demonstrates that basal leaf removal did not significantly alter berry phenology and hence, did not significantly affect berry development and maturity. Moving forward to the next phase of the research, we were able to confidently extrapolate that the differences observed in amino acid accumulation arose from the effect of our experimental leaf removal treatments and not from negative effects on berry development.

7.1.2 Berry pigmentation induced by light exposure

One of the more obvious observations in our study was the change in physical appearance of the berries from leaf removal treatments, in the form of pigmentation in localised spots on the skins of the grapes (Figure 3.3). Grapes berries within the shade of the leaf canopy in control treatments showed minimal pigmentation. This pigmentation only appeared from veraison onwards, despite the fact the berries in the preveraison leaf removal treatments having been exposed to increased light and UV radiation for several weeks prior to veraison. Any examination of biochemical composition of the compounds responsible for the pigmentation spots was beyond the scope of the current study and was therefore, not further pursued.

Nevertheless, our observations are consistent with previous studies in Sauvignon blanc (Gregan et al. 2012; Liu et al. 2015). Liu et al. (2015) also showed that grape bunches exposed to light through leaf removal were clearly pigmented, while grape bunches protected from UV-B wavelengths specifically (using plastic screening techniques), showed a non-pigmented appearance. The indication was that pigmentation was induced by UV-B exposure and the induction was developmental stage specific. UV-B exposure has been shown to increase flavonoid accumulation in a wide range of plant species, including grapes (Jordan 1996; Kolb et al. 2003; Gregan et al. 2012). In addition, *VvFLS4*, which was used in this study as a positive control for leaf removal (and light exposure), has been shown to be regulated by transcription factors active in flavonoid pathways (Liu et al. 2015). The relationship between flavonoid induction and berry pigmentation spots is currently unknown and the mechanism for this UV-

B induced response remains to be determined. However, a number of hypotheses have emerged from other studies and include; the degradation of chlorophyll by long-term exposure to UV-B radiation (Downey et al. 2004; Gonzalez-Barrio et al. 2005); and after UV-stress damage, the activity of the phenolic-related oxidative enzymes, peroxidase and polyphenol oxidase (Gonzalez-Barrio et al. 2005; Rathjen & Robinson 1992).

7.1.3 Factors affecting bunch microclimate

The influence that leaf removal had on bunch microclimate was investigated in the current study. Basal leaf removal exposed the grape bunches to direct sunlight and was shown to increase the amount of radiant heat reaching the fruit. Temperature microloggers placed alongside grape bunches overall showed small average temperature increases comparing leaf removal (exposed bunches and microloggers) treatments with the full leaf canopy controls. The results clearly demonstrated the increases in temperature were due to direct sunlight exposure on the microloggers during the middle part of the day. This is consistent with previous research demonstrating the implementation of leaf removal at the fruiting zone has also been shown to modify temperatures within a grapevine canopy and around the grape bunches (Azuma et al. 2012; Gregan et al. 2012; Haselgrove & Botting 2000).

Other bunch microclimate factors such as airflow penetration and humidity were not quantified in our study, although these parameters would definitely have been impacted by the leaf removal treatments (Austin et al. 2011; Friedel et al. 2015). One of the reasons why vineyard managements frequently involve removing portions of the leaf canopy, is to reduce humidity and increase light exposure to lower disease pressure (Downey et al. 2004; Pereira et al. 2006; Sternad Lemut et al. 2015). Excessive vegetative growth and increased shading can have detrimental effects on the fruit by increasing humidity and therefore disease potential from fungal pathogens for example (English et al. 1990).

7.1.4 Implications of leaf removal for berry biochemistry

Leaf removal doesn't only modify photosynthetic capacity and source/sink partitioning balances of the grapevine, it also considerably alters the grape bunch microclimate. The changes observed in bunch microclimate in our experiments were shown to not significantly impact the berry development parameters monitored. Nevertheless, additional to the aforementioned factors, depending on the specific compounds being measured, the increase in light exposure and bunch temperature, can modify the grape skins biochemistry and influence the overall biochemistry and chemical composition of the grapes (Downey et al. 2004; Haselgrove et al. 2000; Koyama et al. 2012; Liu et al. 2015). The implications of leaf removal and light exposure on the grape bunches is further discussed below in Chapter 7.2 with respect to amino acid accumulation in the grape berry.

7.2 Leaf removal and the light environment can modify amino acid accumulation in Sauvignon blanc grapes

Given that leaf removal is performed routinely in commercial vineyards, we used this common viticultural intervention as an experimental technique to investigate the effects on amino acid biochemistry in Sauvignon blanc grapes. The amino acid composition in Sauvignon blanc grape berries was shown to change significantly through development. Our results reflected changes in total amino acid concentrations in the berry and included differential accumulation at the level of amino acid families and individual amino acids (see Chapter 4). Both preveraison and postveraison leaf removal was shown to significantly reduce total amino acid accumulation in Sauvignon blanc grape berries and again, this was further quantified to differences at the individual amino acid level. While we consider that the significant reduction of amino acids with basal leaf removal is due to disruption of source/sink balances and partitioning influences (discussed further in Chapter 7.3), the impact of increased light and UV radiation exposure has been specifically investigated in other studies, as key factors for influencing amino acid concentrations in grapes.

As discussed above with respect to berry phenology measurements, a direct result of basal leaf removal is to expose the grape bunches to increasing light and higher levels of UV radiation. Interpreting the consequences that the amount and quality of light (solar radiation) exerts on berry composition and biochemistry are difficult to elucidate. The influence on grape composition may result from the direct impact on berry metabolism (light regulated gene expression, for example), or the indirect influence that exposure to light has on overall leaf canopy functions (photosynthetic activity or partitioning effects, for example). Additionally, it can be difficult to unravel temperature (radiant heat) effects from increased UV radiation/light exposure-driven regulation.

7.2.1 Amino acids and the light environment - the role of UV

The light environment is known to regulate the biosynthesis of amino acids in many plants. For example, experiments in Arabidopsis have shown that leaves can differentially accumulate particular amino acids depending on production in the light or in the dark (Coruzzi et al. 2015). Specifically, UV-B wavelengths have been reported to impact nitrogen metabolism by affecting the activity of nitrogen assimilating enzymes (Dohler et al. 1995; Singh et al. 2012). Whether or not such light related studies can translate to amino acid composition in grapevine and specifically, amino acid accumulation in the grape berry has remained a question for further examination.

The role of light and UV radiation in determining amino acid composition has previously been investigated by our research group (Gregan et al. 2012). In this earlier study which spanned three seasons of vineyard (field) experiments in Sauvignon blanc berries, amino acid concentrations were

shown to be significantly decreased by basal leaf removal. There was no additional effect of UV radiation exclusion (using plastic screening) on amino acid composition, compared to leaf removal alone (Gregan et al. 2012). Assuming the removed leaves were a significant source of assimilates, this indicates that leaf removal had a more significant effect on amino acids than changes to UV-B exposure of the grapes. On this point, what was very clear in these experiments was a UV-B specific induction of flavonoid production and up-regulation of associated flavonoid biosynthetic and regulatory genes (Gregan et al. 2012; Liu et al. 2015). The same observations were not noted with respect to amino acid metabolism and UV regulation.

Earlier studies of UV radiation and amino acid composition in grape berries offered up conflicting results (Keller & Torres-Martinez 2004; Schultz et al. 1998). Schultz et al. (1998) used screens to compare ambient UV-B with a reduction to 10% of ambient and observed a reduction of total amino acid concentrations in Riesling must with UV-B exposure. The authors attributed this to a direct effect by UV-B on key enzymes of nitrogen metabolism. In contrast, Keller and Torres-Martinez (2004) demonstrated no effect of UV-B radiation on total amino acid concentration using potted Chardonnay and Sauvignon blanc vines for their study. Results from more recent studies are in agreement in Keller and Torres-Martinez (2004) and indicate a minimal role for UV-B affecting amino acid accumulation in grape berries (Gregan et al. 2012; Martinez-Lüscher et al. 2014). Using controlled environment experiments where the whole potted vines undergo differing light (UV) treatments, showed no effect of UV-B radiation on total amino acid concentrations in the pulp (Martinez-Luscher et al. 2014). This later study however, did note some influence of UV-B on metabolism at the individual amino acid level.

7.2.2 Amino acids and the light environment - cluster shading

In the absence of leaf removal and therefore significant modifications to source/sink balances and partitioning influences, shading of grape clusters can affect amino acids concentrations in berries, compared with berries from naturally exposed bunches. This differential influence by cluster shading has been shown in several studies and suggests that the response of amino acid accumulation to light exposure depends on grape variety and berry tissue type (Friedel et al. 2015; Guan et al. 2017; Martin et al. 2016; Pereira et al. 2006). An important factor to note, is that the above studies using artificial shading techniques, do not address the considerable issues of altering major developmental light signals and the subsequent downstream effects on berry amino acid metabolism. Changes to diurnal cues for example, have been shown to affect both amino acid concentrations in grapes and regulation of amino acid metabolic genes (Rienth et al. 2014; Stitt et al. 2002; Wang et al. 2014).

Using opaque boxes to shade Gamay Noir and Gamay Freaux grape clusters, Guan et al. (2017) showed a decrease of total amino acid concentrations in the berry skins compared to skins from exposed clusters. Additionally, the influence of shading on pulp amino acid concentrations was not as significant.

The skin and pulp of Cabernet Sauvignon has also been shown to respond differentially to light exposure, with shaded berries accumulating less total amino acids (Pereira et al. 2006). However in their experimental set-up, Pereira et al. (2006) used different levels of leaf removal to achieve their shading treatments and also concluded that it was not possible to separate light effects from temperature and leaf area effects. The results obtained from cluster shading experiments might be cultivar specific, with several studies showing that white varieties respond oppositely. Friedel et al. (2015) showed that bunch shading of Riesling grapes increased amino acid concentrations. In addition to bunch shading, this study also used leaf removal treatments, which demonstrated a reduction in amino acid concentrations compared to the shaded and control treatments. In a three year study investigating shading in Sauvignon blanc grapes, light exposed berries accumulated increased total amino acids than shaded berries in one year, but did have less total amino acid concentrations in the second and third years (Martin et al. 2016).

7.2.3 The leaf canopy is important for accumulation of amino acids in grapes

In this current study we didn't investigate the direct effects of the altered light environment (with our leaf removal treatments) and the impacts on amino acid accumulation in the grape berry, but we have previously addressed the role of light and UV radiation in determining berry composition using a similar experimental set-up in previous publications (Gregan et al. 2012; Liu et al. 2015). In vines with a minimally modified canopy and maintained leaf area, a number of studies (discussed above) point to (natural and artificial) shading of grape bunches in having an effect in modifying amino acid in the berries, compared to berries from naturally exposed bunches. In addition to this, the direct evidence for UV wavelengths of light specifically having a role in modifying amino acid accumulation in the grape berry is minimal.

Therefore, while light exposure and shading of fruit can modify amino acid accumulation in grape berries, we suggest that the significant reduction in amino acid accumulation in our current study is predominantly due to the effect of an altered source/sink and partitioning impacts. In our study, we consider the significant loss of a basal leaf source to supply assimilates in the berry (sink) overrides light induced changes with exposure after basal leaf removal. This is further discussed in Chapter 7.3.

7.3 Leaf removal, source/sink modifications and partitioning influences

The results obtained in this current study suggest that the major quantitative effect of basal leaf removal reducing amino acid accumulation in Sauvignon blanc grapes, is due to disruption of source/sink balances and partitioning influences, as opposed to a direct consequence of other parameters such as light or temperature. The results from Friedel et al. (2015) also indicated that leaf removal factors other than light, had a greater influence on amino acid accumulation in grape berries. But as mentioned above, the difficulty in separating out the overlapping side-effects from leaf removal such as changes in light exposure and temperature can lead to contrasting results (Pereira et al. 2006).

Nevertheless, the studies in which basal leaf removal was a primary experimental treatment, indicate the considerable influence of source/sink influences in determining amino acid accumulation in grape berries (Friedel et al. 2015; Gregan et al. 2012). Our study utilised two timings of basal leaf removal, a preveraison treatment and a postveraison treatment. The reductions in total amino acid accumulation in the grape berry after leaf removal, occurred promptly following application of the preveraison and postveraison leaf removal treatments. This demonstrates a relatively immediate effect of leaf removal, and could indicate a sudden decrease in the import of assimilated nitrogen (amino acids) being contributed by the proximal basal leaves around the grape bunches. This is in contrast to TSS accumulation in the grapes whereby, °Brix levels were relatively unaffected by basal leaf removal. The relevance of these observations to partitioning influences, nitrogen assimilation and leaf aging are discussed below.

7.3.1 Partitioning

Crucially as seen in our experiments, basal leaf removal had a significant effect on amino acid accumulation in the berries, whereas the effect on TSS accumulation was minimal. As discussed above in Chapter 7.1.1 (also see Figure 3.1 and Figure 3.4), the allocation of assimilates to be exported to the sink tissues is called partitioning with the basal leaves preferentially supplying the grape bunches, especially as the fruit matures and becomes a strong and dominant sink for assimilates (Wardlaw 1990). As any source modification such as our basal leaf removal treatments, force the more distal leaves on the shoot to export a greater proportion of their assimilates to the clusters to compensate for this loss (Quinlan & Weaver 1970). It is therefore likely that the import of amino acids into the berry is impaired by basal leaf removal and that the more distal leaves on the shoots and laterals try to compensate to some extent for the loss of the basal leaves. But due to other effects such as younger leaves and competition from other sinks (such as growing shoot tips and redistribution into perennial parts of the vine), this compensation is not achieved, and hence the reduction in amino acid accumulation. The

allocation and transport of carbohydrate and accumulation of sugars in the berry is seemingly less impacted by the same partitioning influences as shown in our experiments (Figure 7.1) (Poni et al. 2018).

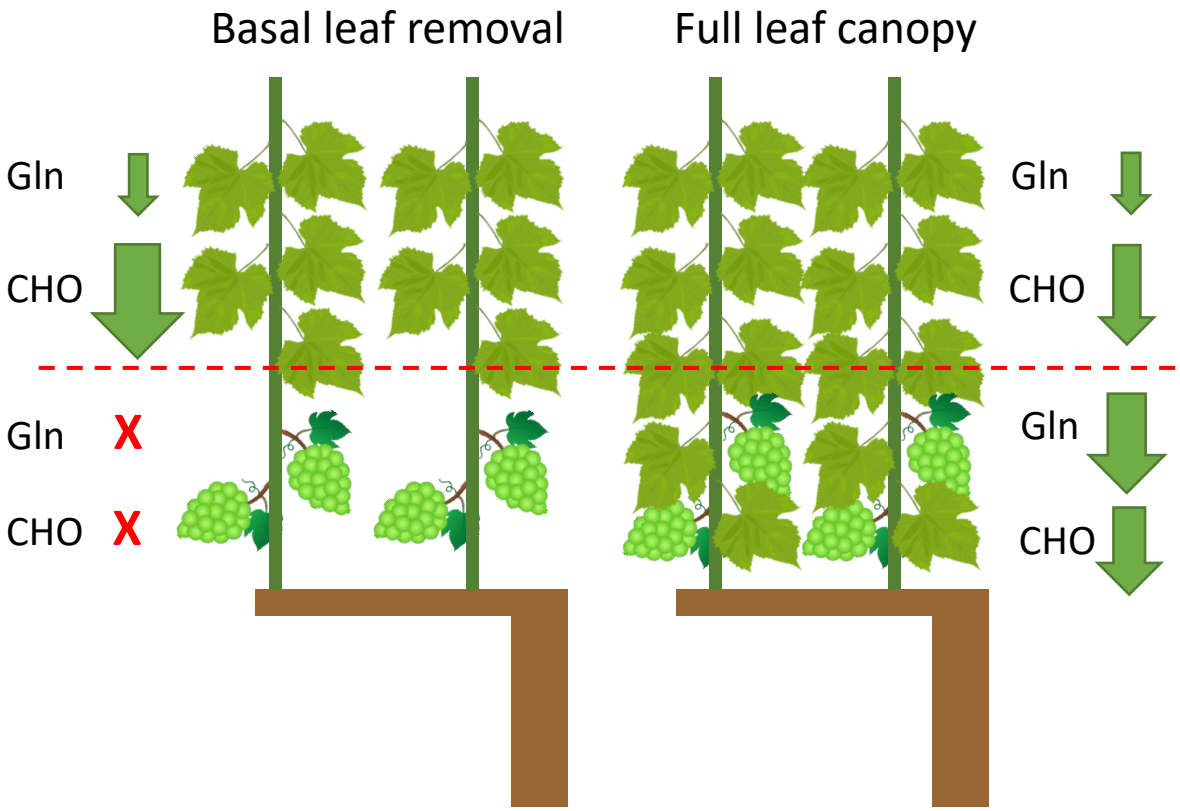


Figure 7.1 A proposed model to describe how basal leaf removal may have differential effects on amino acid and carbohydrate export into grape bunches.

Leaves around the grape bunches are mature enough to export amino acids (glutamine, Gln) and carbohydrates (CHO). Removal of the basal leaves may influence the more distal leaves on the shoot to compensate for this local loss. The net result is that the distal leaves may not be at full capability to export significant quantities of nitrogen compounds but are still able to export appreciable amounts carbohydrates.

7.3.2 Nitrogen assimilation and leaf aging

Basal leaf removal therefore significantly reduces total amino acid concentrations in Sauvignon blanc grape berries and this reduction is also reflected in the nitrogen assimilation amino acids, glutamine and glutamate. Additionally to partitioning influences, an important point in the context of this current study, is that throughout leaf development and aging, there is a progression in the types of compounds exported, from sucrose early in development to predominantly amino acids and other nutrients (Masclaux-Daubresse et al. 2010; Thomas 2013). The basal leaves around the grape cluster are the older leaves of the shoot and before application of the leaf removal treatments, are presumably mature enough to export amino acids to the grape bunches. This was seen with the high concentrations of

glutamine in all treatments preveraison. Removing these basal leaves likely caused the vines ability to export amino acids into the grape bunches to be impaired. This could explain why disruption to the source/sink balance and partitioning of the grapevine influenced the amino acid and TSS accumulation differentially in the grapes. The younger, but more distal leaves were presumably not at full capability to export significant quantities of nitrogen compounds but were still able to export appreciable amounts carbohydrates (Figure 7.1) (Diaz et al. 2005). Hence, amino acid accumulation was decreased in the grape berries but accumulation of TSS was affected only minimally. An aging leaf therefore maintains its role as a source and significant exporter of nutrients, but the type of compounds change to be mostly nitrogenous instead of carbon (carbohydrate) rich (Reich et al. 2009).

The nature of compounds exported through leaf development approaching senescence, is therefore consistent with the results observed in our current study. As previously mentioned, a prerequisite to nitrogen assimilation in the leaves is a sufficient photosynthetic capacity for energy and a supply of carbon backbones through the TCA cycle. Nitrogen assimilation in newly emerging leaves is therefore limited, but proceeds rapidly in more mature leaves with a high carbohydrate status and photosynthetic activity (Perez & Kliewer 1982). Once amino acids start accumulating in the leaves, they are transported to leaf nitrogen storage pools. As the leaves age, experiments in *Arabidopsis* have shown that these stores are exported to sinks, with amino acid concentrations decreasing in the leaves while increasing in the phloem sap (Have et al. 2017). The same observations have been noted in grapevines (Konstantinos A. Loulakakis et al. 2002). As leaves age further and during senescence in particular, leaves transition to become considerably strong sources for amino acids (White et al. 2016). Senescence mechanisms enable plants to remobilise and redistribute the significant nutrients accumulated, for example, proteins represent the largest nitrogen pools in every plant tissue. Senescence is also accompanied by varied and complex mechanisms for nitrogen remobilisation such as amino acid transporters, proteolytic mechanisms and a significant role for aminotransferase enzymes (Diaz et al. 2005; Forde & Lea 2007; Have et al. 2017).

7.3.3 Qualitative aspects of amino acid accumulation in grapes

Basal leaf removal was also shown to have a qualitative effect on the proportions of amino acids that accumulated in Sauvignon blanc grape berries, with respect to the leaf removal treatments. The α-ketoglutarate family of amino acids together, showed greater proportions with basal leaf removal in both seasons at postveraison time points (Figure 4.4). Consequently, the other amino acids measured contained lower proportions of amino acids postveraison with leaf removal compared to the CANOPY controls. We therefore investigated mechanisms that could affect both quantitative and qualitative aspects of amino acid metabolism by examining transcriptional changes of a number of genes involved in biosynthesis, degradation and regulation of the α-ketoglutarate family of amino acids (Chapter 5).

We also investigated OAT protein enzyme activity to further examine the mechanisms of amino acid accumulation in the α-ketoglutarate family (Chapter 6). These are discussed further in Chapters 7.4 and 7.5.

Additionally, after import into the berry, glutamine/glutamate concentrations in the berry are likely converted to other amino acids through the actions of aminotransferases or as direct precursors (Forde & Lea 2007). Combine this with a leaf removal induced reduction of glutamine and glutamate from nitrogen assimilation mechanisms, and it seems sensible to suggest that there will be effects in the modification of downstream amino acid metabolic pathways contributing to both quantitative and qualitative consequences. But in general, with the large number of amino acids, the broad range of their composition and concentrations within cells and tissues and the fact that their diverse biosynthesis and transport mechanisms are just starting to be fully realised, means there is considerable scope for qualitative aspects of amino acid accumulation to be modified (Forde & Lea 2007; Hachiya & Sakakibara 2017; Tegeder & Masclaux-Daubresse 2017). We have also discussed the role of light and UV radiation effects on qualitative aspects of amino acid accumulation in grape berries (Guan et al. 2017; Martinez-Luscher et al. 2014). The complex mechanisms involved was beyond the scope of the current study but some important progress has been made in recent years.

There is limited research about transporters involved in amino acid export from leaves, but more recent research has identified several candidates that play a role in amino acid phloem loading (Tegeder 2014). Additionally, several senescence induced amino acid transporters have been identified, but their role in remobilisation of nutrients has not been further characterised (Have et al. 2017). Studies in Arabidopsis and peas have shown that amino acid transport mechanisms in the leaves can exert control over nitrogen uptake in the roots. The same amino acid transport function can also influence leaf metabolism and partitioning to sinks (Santiago & Tegeder 2016; Tan et al. 2008; Zhang et al. 2015). As well as amino acid transport from the leaves, regulatory aspects of nitrogen assimilation and nitrogen/nitrate sensing and signalling are proving critical for plant performance, the mechanisms of which are just starting to be unravelled (Gent & Forde 2017; Jacquot et al. 2017).

7.4 The α-ketoglutarate amino acids

7.4.1 Glutamine and glutamate accumulation and leaf removal

We found that in preveraison Sauvignon blanc berries and before application of leaf removal treatments, substantial concentrations of glutamine had already been accumulated and contributed over 70% of total amino acids in both seasons experiments (Figure 4.5). Glutamine is therefore, clearly the predominant amino acid present early in grape development and is compatible with glutamine having shown to be the major nitrogen transport compound into grape berries. Consistent with our results, glutamine is reported to be the major amino acid exported to the berry in grapevine xylem and phloem sap at preveraison developmental stages (Andersen & Brodbeck 1989; Glad et al. 1992, 1994; Peuke 2000). The import of nitrogen compounds into the grape berry after veraison is restricted to the phloem, but has also been shown to be mostly in the form of glutamine (Gholami 1996; Glad et al. 1992). However, smaller amounts of other amino acids have shown to be present in the phloem sap of grapevines including glutamate, aspartate, proline and arginine (Gholami 1996; Glad et al. 1992). The proportions of each amino acid appear to vary through development and depending on the cultivar, but grape variety aside, glutamine is shown to predominate at all stages.

Glutamine concentrations were shown to decrease significantly through development, with the rate of decline greatest at preveraison timepoints. The only other amino acid to show a similar decreasing concentration profile throughout development was asparagine. The other amino acids analysed exhibit steady increases throughout ripening or don't show an overall decrease. Even combined with the decrease in glutamine concentrations in CANOPY control berries (with a maintained leaf canopy) through development, our results also demonstrate a significant reduction of glutamine in the berry following basal leaf removal in the PRE and POST treatments. Similar observations of glutamine predominating in preveraison berries and declining through development have also been shown by a number of studies in grapevine specifically (Friedel et al. 2015; Gregan et al. 2012; Rienth et al. 2014; Stines et al. 2000).

The steady decrease of glutamine concentrations is likely to be the result of the conversion into other amino acids in the grape berry. It is also probable that this is in conjunction with glutamate through the activities of the GS/GOGAT pathway (Loulakakis et al. 2009) and that glutamine is metabolised at a faster rate that it can be imported or synthesised internally. Another suggestion for declining concentrations of glutamine could be that the rate of transport into the berry decreases through development. The transport mechanisms of amino acids into the berry were not addressed in our study, however this is probably unlikely, due to the remobilisation and considerable export of nutrients that takes place during leaf aging and senescence (as discussed above in Chaper 7.3.2). Additionally to this point, we consider that it is the removal of these basal leaves with a significant capability for amino acid export that

contributes to the reduction of glutamine concentrations in the berries from our leaf removal treatments.

In direct contrast to glutamine, concentrations of glutamate are considerably lower at preveraison time points, but it is still the second most abundant amino acid preveraison after glutamine. In general, concentrations of glutamate are remarkably consistent in comparison with the other amino acids. This is particularly interesting as glutamate is the direct precursor of the two most abundant amino acids (arginine and proline) which are increasing through postveraison time points, and the primary source of amide groups for other amino acids via transamination reactions (Forde & Lea 2007; Majumdar et al. 2016; Stines et al. 1999). This raises the question of, how and why glutamate concentrations are maintained with relatively consistent levels, while the concentrations of the other amino acids change so considerably. This effect of glutamate concentrations remaining relatively constant in response to major developmental and external cues has been noted by a number of studies (Fritz et al. 2006; Novitskaya et al. 2002; Stitt et al. 2002). In these studies, the concentration of most amino acids varied substantially, while the concentration of glutamate notes only minor fluctuations. Indeed a number of reviews have highlighted the importance and central role of glutamate in plant nitrogen metabolism (Bouche & Fromm 2004; Forde & Lea 2007; Hachiya & Sakakibara 2017; Tegeder & Masclaux-Daubresse 2017).

It therefore seems, in our experiments in Sauvignon blanc grape berries, the maintenance of glutamate levels is important for berry homeostasis and along with glutamine, as a metabolite in a wide variety of biochemical pathways. While small amounts of glutamate may be imported into the berry directly from the phloem, it is probable that the majority of glutamate present in the berry is produced from other sources. The dominant pathway is most likely to be through the actions of the GS/GOGAT pathway in conjunction with glutamine. Therefore, the reduction of glutamate following basal leaf removal is presumably occurring as a follow on effect of decreased glutamine concentrations (from basal leaf removal) and the reduced flux of molecules through the GS/GOGAT enzymes. Nevertheless, while glutamate concentrations in berries from the leaf removal treatments are also decreased, they do also manage to maintain relatively consistent concentrations through development.

7.4.2 Mechanisms of glutamine and glutamate accumulation - GS and GOGAT

The reduction of glutamine and glutamate due to basal leaf removal, demonstrated both a quantitative and qualitative modification of their concentrations in the grape berry. Therefore, for a greater understanding of the mechanisms involved in glutamine and glutamate accumulation in grapes, we investigated expression in the berry of several isoforms of genes involved in nitrogen assimilation in grapevine; three isoforms of *GS1* (*GS1-1, GS1-2* and *GS1-3*) and two isoforms of *GOGAT* (*NADH-GOGAT-1* and *-2*).

nCounter analysis of the *GS1* isoforms demonstrated relatively high transcript counts of *GS1-1* and a dramatic increase in *GS1-1* transcripts at postveraison timepoints. Whereas, *GS1-2* and *GS1-3* showed the highest transcript levels at preveraison time points and had considerably less transcript counts than *GS1-1* at all stages of development. The activity of proteins made from these transcripts was not determined in this study, although the results do indicate that the *GS1-1* transcript is potentially a major contributor to the cytosolic *GS* isoenzymes in grape berries at all stages of development. The *GS1-2* and *GS1-3* transcripts may have a role in preveraison berries. Two isoforms of *NADH-GOGAT* were also analysed by nCounter, *NADH-GOGAT-1* and *NADH-GOGAT-2*. *NADH-GOGAT*-1 was found to have constitutive expression with consistent and relatively high transcript counts throughout development. *NADH-GOGAT-2* contrastingly, had extremely low (barely detectable) transcript abundance and therefore was considered to be not expressed at any stage of development measured. The result of *NADH-GOGAT-1* having high and constitutive expression was not unexpected, as this enzyme presumably works in conjunction with GS to maintain glutamate levels in the berry. The expression pattern of *NADH-GOGAT-1* would fit this model and indicates that the *NADH-GOGAT-1* transcript could contribute to the GS/GOGAT pathway in grape berries. There were no differences on the *GS1* or *NADH-GOGAT-1* transcript counts induced by basal leaf removal. This indicates that the quantitative and qualitative differences seen in glutamate and glutamine concentrations are likely not due to differential expression of the *GS1* and *NADH-GOGAT* transcripts.

Previous studies have demonstrated expression and enzyme activity of the cytosolic *GS1* isoforms in grapevine tissue (Loulakakis & Roubelakis-Angelakis 1996; Paczek et al. 2002). Our results are supported by these earlier reports of differential tissue expression of the *GS1* isoforms, with *GS1-1* highly expressed in berry tissue and significantly lower expression of *GS1-2* in berries. *GS1-3* expression has not been previously reported in grape berries (Loulakakis et al. 2009; Loulakakis & Roubelakis-Angelakis 1996). NADH-GOGAT activity has been detected in a number of grapevine tissues including roots, leaves and cultured cells, however, the activity of NADH-GOGAT has not yet been demonstrated in grape berries (Loulakakis & Roubelakis-Angelakis 1997). A previous study did demonstrate expression of two transcripts in grape berries which correspond to the NADH-GOGAT-1 and NADH-GOGAT-2 isogenes analysed here and in contrast to our results, show expression of both isoforms in grapes and a down-regulation in ripening berries (Rienth et al. 2014). Their experimental set-up was comparatively different to ours, using microvines in fully controlled environments, which could in some part explain the differences in expression patterns seen.

The increasing expression of *GS1-1* and constitutive expression of *NADH-GOGAT1* in our study, could be a mechanism by the grape berry to bolster GS1 and GOGAT protein levels for maintenance of glutamate levels, as glutamine concentrations in the grape berry are diminished. A number of other observations from previous studies could contribute to the quantitative and qualitative changes of glutamine and

glutamate accumulation in grape berries, as seen in our study. For example, Famiani et al. (2000) showed an abundance of GS1 protein in grape berries and other tissues associated with assimilate transfer. Further to this point, changes in the content of GS1 and GOGAT isoforms in non-leaf sink tissues have been linked to maintenance of glutamate levels, from imported glutamine specifically (Hayakawa et al. 1994; Tabuchi et al. 2007). GS1 has also been proposed to play a central role in proteolytic nitrogen remobilisation and is enhanced during the recycling of organic nitrogen released from protein degradation (Paczek et al. 2002). Additionally, in potential allosteric regulation, the presence of glutamine in the medium of grapevine cell cultures causes a decrease in both protein level and enzyme activity of GS1 isoforms (Loulakakis & Roubelakis-Angelakis 1996).

7.4.3 Proline and Arginine accumulation and leaf removal

In our study we have shown that the two most predominant amino acids in mature Sauvignon blanc grape berries were proline and arginine. This is consistent with many previous studies investigating amino acid accumulation in a variety of different grapevine cultivars (Gregan et al. 2012; Stines et al. 2000; van Heeswijck et al. 2001). In addition, both proline and arginine concentrations were shown to be decreased significantly at postveraison stages of berry development following basal leaf removal treatments.

As glutamate is the direct precursor of both of these amino acids, it is likely that the reduction of proline and arginine following basal leaf removal potentially occurs from the decreased flux of glutamine and glutamate (Chapter 7.4.1) through the GS/GOGAT pathway induced by the same basal leaf removal treatments. However, our observations do not rule out the possibility of partitioning effects and leaf removal influencing the import of smaller amounts of proline and arginine into the grape berry from a (modified) leaf source. But as discussed in Chapter 7.4.1, although proline and arginine have been previously shown to be present in the phloem sap, they are in small concentrations compared to the predominant glutamine. Therefore, the major effect of leaf removal on proline and arginine concentrations is likely to be from a reduction of glutamine import and the subsequent reduction of flow of glutamate into the proline and arginine biosynthetic pathways. Another point of regulation for reduced proline and arginine concentrations in the berries could be a change in expression of biosynthetic transcripts, this is further discussed below in Chapter 7.4.4 and 7.4.5.

Previous studies which have examined different grape cultivars grown in similar soil and climatic conditions have confirmed that amino acid profiles vary significantly between grape varieties, however proline and arginine almost always are present in the greatest concentrations through later berry development approaching maturity (Huang & Ough 1991; Kliewer & Ough 1970; Stines et al. 2000). The absolute concentrations of most amino acids differs considerably between studies and cultivars, however the ratios of proline to arginine can remain reasonably constant for certain cultivars (van

Heeswijck et al. 2001). This would suggest that the general profiles of amino acid accumulation is predetermined within each cultivar, with other variables (environmental, canopy management, for example) contributing only a modifying effect.

As discussed in Chapter 1, much of the research examining proline metabolism in other plants have focussed on its accumulation in response to abiotic stresses such as drought and salinity (Heuer 2010). However, the role of proline as an abiotic stress response in grapes is so far undefined. As has also been shown in other studies, the results presented here demonstrate that the pattern of proline accumulation is non-uniform over the course of berry development and is mainly occurring at postveraison stages (Gregan et al. 2012; Stines et al. 1999). The inference is that the mechanisms of proline accumulation in grape berries are separate from those operating during abiotic stress responses in other plants (Kavi Kishor & Sreenivasulu 2013). Arginine on the other hand, is considered as a major storage and transport form for organic nitrogen in plants in addition to its other roles in metabolic and cellular processes (Winter et al. (2015) and references within). This is also likely to be the case in developing grape berries given that is easily the predominant amino acid at postveraison stages of development (see Table 4.3). Therefore, the accumulation of arginine and its subsequent metabolism, is likely to play an important role in nitrogen storage, recycling and redistribution in grape berries (Slocum 2005).

We suggest that what may be more important in grape berries, is the ability of proline and arginine to preserve a central role in amino acid metabolic pathways, the flux through these pathways contributing to the maintenance of cellular homeostasis. Far from being a passive accumulation of proline and arginine in the grape berry, we have shown that the genes involved in both proline and arginine degradation have increased expression through development, therefore potentially contributing to a significant turnover of these amino acids even as they are being synthesised and continuing to accumulate (see Chapter 7.4.4 and 7.4.5 below). So even though the berries are contributing considerable resources into accumulating both proline and arginine, this is seemingly a dynamic process with the increased expression of genes in degradation pathways potentially playing a role during berry development. As discussed in Chapter 7.4.1, one of the end goals of the dynamic processes of proline and arginine metabolism in grape berries, could be to maintain concentrations of glutamate, given its central role in plant nitrogen metabolism and as glutamine levels in the berry are decreasing. This point is further discussed in Chapter 7.5.

The net accumulation of proline and arginine in grape berries is therefore, the result of the balance between synthesis and degradation. In grape berries, the turnover of proline in the mitochondria might also be linked to a role in oxidative respiration and a supplementation of energy generation (Hare & Cress 1997; Kavi Kishor et al. 2005). Maintaining a balance between synthesis and degradation or proline

for example, has been shown to be vital to a favourable redox balance ($NADP^+$/NADPH ratio) and detoxifying reactive oxygen species (Miller et al. 2009; Sharma et al. 2011). Specifically in grapevine, accumulation of proline in leaves has been shown to have a direct positive effect on mitigating oxidative stress (Ozden et al. 2009; Skopelitis et al. 2006). But whether or not the accumulation of proline in grape berries plays a role in antioxidant enzyme mechanisms remains a question for further investigation.

7.4.4 Mechanisms of proline accumulation – proline biosynthetic genes

To examine the mechanisms of proline accumulation and degradation in Sauvignon blanc grape berries, we investigated the expression of transcripts potentially involved in its biosynthetic and catabolic pathways. Similarly to glutamine and glutamate, proline demonstrated both a quantitative and qualitative modification of its concentrations in the grape berry, of which changes in expression of metabolic transcripts could at least in some part account for these observations. nCounter analysis of the biosynthetic transcripts in grape tissue, showed that *P5CS* is highly (and constitutively) expressed throughout berry development and increases through veraison and postveraison time points. This does overlap the period in berry development when proline accumulation in the berry is increasing and the berry is starting to accumulate larger amounts of sugars. However, leaf removal does not affect the transcript counts of *P5CS* which suggests that differential expression of this proline biosynthetic gene is not responsible for changes observed in proline concentrations in the grape berry (quantitative and qualitative).

As discussed in Chapter 1.7, in many plant species *P5CS* is encoded by two genes, one of which can be induced by stress mechanisms. While *VvP55CS* has previously been shown to be only one gene in the grapevine genome (Stines et al. 1999), a relatively recent report detected expression of two putative *P5CS* isogenes up-regulated in ripening berries (Rienth et al. 2014). It is therefore possible that these additional *P5CS* transcripts may play a role in proline accumulation as an additional stress/developmental response. However, we were unable to detect expression of these two transcripts at any stages of berry development measured (Chapter 5.5.1). There have been no new studies or any further characterisation of these transcripts reported in the literature.

Previous reports of *P5CS* in grape berries showed that steady-state levels of mRNA remain relatively constant through development, with only transient increases at 4 and 12 weeks postflowering (Stines et al. 1999; van Heeswijck et al. 2001). The authors suggested that the accumulation of proline during berry development is independent of changes to P5CS expression and protein levels. Conversely, we show a significant increase in *P5CS* transcript counts through veraison and a maintenance of transcript counts at postveraison time points. Assuming concomitant increases in P5CS protein levels, transcription of this single gene in grapevine may be sufficient for the grape berry to accumulate adequate proline concentrations. We suggest that the developmental capacity for proline synthesis in

grape berries is maintained by considerable (and increasing) expression of *P5CS*, with other factors, such substrate supply potentially changing the flux through the metabolic pathways and modifying proline concentrations (as observed in our leaf removal experiments). In addition, recombinant grapevine P5CS has been shown to be feedback inhibited and influenced by glutamate concentration (Stines et al. 1999). We show that glutamate concentrations are maintained relatively constantly through berry development, and so therefore may not be present at sufficient concentrations to allosterically regulate the P5CS enzyme and inhibit proline accumulation. Therefore, both of these results provide a potential mechanism for the significant postveraison accumulation of proline in Sauvignon blanc grape berries.

The profile of *P5CR* expression in grapevine (and grape berries) has so far not been reported. Here we show that a putative *P5CR* transcript is constitutively expressed throughout berry development, however, the transcript abundance was significantly lower at all stages of development compared to *P5CS*. This result is consistent with P5CR not being considered to be the rate-limiting step in proline biosynthesis in plants and its function being to support proline biosynthesis, and probably not contributing a significant regulatory role. Although *P5CR* expression has been shown to respond to osmotic pressures in some plants, our results indicate that in grape berries, this is not the case.

7.4.5 Mechanisms of proline accumulation – proline degradation genes

nCounter analysis of a grapevine *PDH* transcript showed that *PDH* transcript counts increase substantially through postveraison time points, overlapping the period in berry development when proline is accumulating in spite of the increased expression of *PDH*. Interestingly, the transcript abundance of *PDH* in the leaf removal treatments was consistently higher at postveraison time points, compared to CANOPY control samples. The relevance of this observation is important, as any changes to the transcript abundance of *PDH* could impact proline concentrations in the grape berry and contribute both to the quantitative and qualitative differences observed in proline accumulation (with respect to the leaf removal treatments). This latter observation would require further investigations to confirm its validity and its potential relevance and contribution to proline concentrations.

Related observations of increased *PDH* expression through berry development have been noted by other studies. Stines et al. (1999) also studied proline degradation in grape berries by investigating the levels of the PDH protein through development. They reported levels of the grapevine PDH protein to be increasing through berry development and deduced that proline accumulation in the grape berry was likely not due to a decrease in proline degradation. No further characterisation of the grapevine PDH, including measurements of enzyme activity have been pursued in the literature. This remains an important area of proline research in grapevine open for further investigation.

As discussed above, the accumulation of proline in grape berries appears to occur through a distinct mechanisms compared to those observed in other plant species during abiotic stress responses. In addition, the profiles of expression of *P5CS* and *PDH* in developing berries are also comparatively distinct to observations from other plants. For example, the response of most plants to osmotic stress, is a decrease of transcription and enzyme activity of PDH and a subsequent increase during stress removal and recovery. These changes in PDH transcription and activity are generally inversely correlated with free proline concentrations (Mattioni et al. 1997; Peng et al. 1996; Rayapati & Stewart 1991). Therefore, the developmentally regulated osmotic changes occurring in grape berries may be distinct and potentially occur relatively gradually, compared to the rapid onset of stress that is imposed on plants due to drought and salinity pressures, for example. There is some precedent for this last point. In *Arabidopsis*, during "normal" (non-stressed) conditions, *PDH* mRNA expression is greatest in tissues which also contain the highest concentrations of proline (Nakashima et al. 1998). This suggests the potential for a high turnover of proline in these tissues and could be complementary to the function of proline catabolism and cellular homeostasis discussed above in Chapter 7.4.3.

There are currently no reports in the literature of *P5CDH* expression specifically in grape berries. nCounter analysis of *P5CDH* in developing Sauvignon blanc grape berries demonstrated a relatively high transcript abundance and constitutive expression compared to the other proline catabolic gene, *PDH*. While not impacted by leaf removal treatments, the considerable expression of the *P5CDH* transcript suggests that P5CDH is likely to be an important enzyme in grape berry for the production of glutamate and maintenance of cellular homeostasis mechanisms (see Chapter 7.4.1 and 7.4.3). Additional to the activity of PDH, OAT catalyses the reaction of ornithine to P5C (the substrate of P5CDH) in the mitochondria (Delauney & Verma 1993; Funck et al. 2008). This separate pathway to P5C production represents an interconnection between proline and arginine metabolism and is further discussed below in Chapter 7.5.

In grape berries therefore, we demonstrate a constitutive high transcript abundance of *P5CDH*, the relative levels of which appear to be unaffected by the proline concentrations in the grape berry. This is distinct to reports from other plants. For example, the *P5CDH* gene has been shown to be expressed at only low basal levels across *Arabidopsis* tissues and can be upregulated by proline (Deuschle et al. 2001). Activation of proline biosynthesis in *Arabidopsis* has previously also been shown to be tightly linked to ABA-mediated signalling (Abraham et al. 2003). A more recent study specifically in grapevine, investigated ABA treatment on ripening berries and demonstrated an additional effect on proline degradation, observing a decrease in the abundance of P5CDH protein following ABA treatment (Giribaldi et al. 2010). The authors suggest that proline accumulation in grape berries could be controlled by an ABA-mediated inactivation of P5CDH, although there are no reports of any further characterisation. Given that the activity of P5CDH in grapes is not specific to only the proline

degradation pathway, it seems unlikely that a significant regulatory point of control of proline accumulation is through the inactivation of this enzyme. Further to this point, a lack of P5CDH activity has been shown to compromise proline degradation mechanisms which leads to increased ROS production because of excess proline accumulation (Miller et al. 2009). Additionally, *Arabidopsis* p5cdh mutants can accumulate toxic levels of P5C, which significantly alters the cellular redox balance (Cecchini et al. 2011).

7.5 Mechanisms of arginine and ornthine metabolism in grapes – arginase, *OAT* and *SNAC2*

As discussed in Chapter 1.9, ornithine provides an interconnection between arginine and proline metabolism (Figure 1.11). Consequently, a number of studies have hypothesised that mobilising the store of accumulated arginine, could contribute to proline biosynthesis via the intermediates ornithine and P5C through the actions of arginase and OAT enzyme activity. The biosynthetic pathway leading to production of P5C from arginine and ornithine is highly expressed in Sauvignon blanc grape berries. nCounter analysis of *Arginase* and *OAT* expression in grape tissue, showed transcript counts increasing significantly through berry development, especially through postveraison time points. Additionally, the transcription factor SNAC2 (an activator of OAT), was also shown in to increase in transcript abundance through berry development. The high levels of expression of genes involved in this biosynthetic pathway demonstrate that they are likely to have a significant role in amino acid metabolism in Sauvignon blanc grape berries.

We suggest that in grape berries, as has been discussed above, is that expression of arginase, *OAT* and *SNAC2* are important in maintaining glutamate concentrations for cellular homeostasis and participation in biosynthetic pathways. Therefore, the production of P5C in grapes through the activities of arginase and OAT, is likely to be metabolised by P5CDH in the mitochondria to glutamate for use in other metabolic pathways. The high constitutive expression of *P5CDH* we demonstrate in grape berries (Figure 5.10) could also contribute to this mechanism of glutamate production. The export of glutamate from the mitochondria has also been shown by previous studies, whereby it can be a substrate for other biosynthetic pathways, including proline (Winter et al. 2015).

While we did not measure ornithine concentrations in this study, ornithine has been shown to be present in grape berries, but at considerably lower concentrations (0.3-1% of total amino acids) compared to the α-ketoglutarate amino acids (Stines et al. 2000). The low concentration of ornithine would be consistent with its role as an intermediate and it potentially operating as a transient molecule in grape berries, having a high rate of turnover in a number of biosynthetic pathways. Additionally, because of compartmentalisation issues, the production of P5C through this pathway (or by any other mechanism), would require transport out of the mitochondria. Although such mechanisms have been

postulated (Miller et al. 2009), direct evidence for the transport of P5C has not been demonstrated and as such, the role of P5C produced in this manner where it could become a direct substrate for proline biosynthesis is so far uncharacterised.

Arginase protein activity has previously been demonstrated in developing grapes, while the expression of *Arginase* mRNA in grape berries has not been reported before (Roubelakis-Angelakis & Kliewer 1981). Previous studies of *OAT* expression and activity in grapes are also very limited. Stines et al. (1999) showed *OAT* expression in a range of grapevine tissues, but found very little expression of *OAT* mRNA in berries. Additionally, a broad transcriptomic study of berry development using a microvine experimental system, identified an *OAT* transcript expressed in grapes (Rienth et al. 2014). Both of these studies suggested a minimal role for OAT in berry metabolism. Conversely, we demonstrate increasing transcript abundance of *arginase* and *OAT* and suggest that expression of these two transcripts, and the activity of OAT (see below), plays a significant role in amino acid metabolism in grape berries. Further to this point, SNAC2 is a transcription factor that has shown to be able to up-regulate expression of OAT in rice, in a mechanism by directly binding to the OAT promoter (Hu et al. 2008; You et al. 2012). While *SNAC2* expression in grape berries has an expression profile very similar to OAT in Sauvignon blanc grapes, further research is required to determine if SNAC2 can directly regulate OAT in grapevine.

7.5.1 The OAT enzyme is active in Sauvignon blanc berries

Results from this current study demonstrates specific activity of OAT, increasing throughout berry development in Sauvignon blanc grapes. This suggests that OAT activity is likely to play a significant role in amino acid metabolism in grape berries. In addition, relative specific activity was increased by basal leaf removal at all time points measured (compared to CANOPY control samples) (Figure 6.3). The relative specific activity of OAT being increased by leaf removal is an interesting observation and suggests the potential for allosteric regulation of this enzyme. This could potentially occur by changes in the flux of molecules through the α-ketoglutarate metabolic pathways due to the effects of leaf removal. In our experiments therefore, lower concentrations of arginine in samples from leaf removal, could potentially result in a reduction of feedback mechanisms through related pathways, subsequently increasing the relative activity of OAT in leaf removed samples. For example, in a related pathway, the grapevine P5CS enzyme has been shown to be feedback inhibited by proline and the level of inhibition is also influenced by the concentration of glutamate (Stines et al. 1999). In *Arabidopsis*, Funck et al. (2008) showed that the activity of OAT was potentially the only exit route of nitrogen coming from the mobilisation of arginine and ornithine, demonstrated by *oat* mutants not being able to use these amino acids as nitrogen sources for growth.

Overall, this again could be a potential mechanism by the berries to try to maintain glutamate concentrations, especially combined with a reduced flux of molecules through the related metabolic

pathways. To back-up our findings in grape berries, it has been suggested from previous studies, that experiments using *Arabidopsis oat* mutants indicate a role for OAT in arginine catabolism and glutamate synthesis, rather than OAT activity contributing directly to proline synthesis (Funck et al. 2008; Kavi Kishor & Sreenivasulu 2013). Additionally, You et al. (2012) propose a limited contribution of OAT activity to proline accumulation in rice. While Stines et al. (1999), did also identify OAT activity in grape berries, conversely to our findings, they detected only very low levels of OAT protein and enzyme activity and were unable to confirm any contribution of OAT activity to maintenance of amino acid levels in grapes.

7.6 Summary of discussion

The results presented in this thesis demonstrate that basal leaf removal significantly reduces total amino acid concentrations in Sauvignon blanc grape berries. In contrast, the effect of leaf removal on the berry phenology parameter of TSS accumulation was minimal. The inference is that the removal of the basal leaves may cause the vines ability to export amino acids into the grape bunches to be impaired. Therefore, disruption to the source/sink balance and partitioning of the grapevine influences primary metabolism (amino acids and TSS accumulation) differentially in the grapes. The younger, but more distal leaves (trying to compensate for the loss of basal leaves) are most probably not at full capability to export significant quantities of nitrogen compounds but are still able to export appreciable amounts carbohydrates. We additionally consider that the significant reduction in amino acid accumulation in our current study is not due to other effects induced by leaf removal, such as increased light exposure on the grape bunches. Basal leaf removal was also shown to have a qualitative effect on the proportions of amino acids that accumulated in Sauvignon blanc grape berries, with respect to the leaf removal treatments. In particular, the α-ketoglutarate family of amino acids together, showed greater proportions with basal leaf removal in both seasons at postveraison time points.

We therefore surmise that the flux of the α-ketoglutarate family of amino acids through their metabolic pathways potentially contributes to the maintenance of cellular homeostasis and as a mechanism to replenish concentrations of glutamate (given its central role in plant nitrogen metabolism), as glutamine levels in the berry are decreasing. The accumulation and degradation of the α-ketoglutarate amino acids was investigated through expression of transcripts potentially involved in their biosynthetic and catabolic pathways. We have shown that the genes involved in both proline and arginine degradation have increased expression through development, therefore potentially contributing to a significant turnover of these amino acids even as they are being synthesised and continuing to accumulate. Therefore, these pathways can be considered dynamic processes with the increased expression of genes in degradation pathways also playing an important role during berry development. We also show that relative specific activity of OAT is increased by leaf removal, suggesting the potential of allosteric regulation of this enzyme.

7.7 Concluding remarks

The research in this thesis enhances our knowledge of the mechanisms influencing amino acid biochemistry in *Vitis vinifera* L. var. Sauvignon blanc grape berries.

The following objectives were addressed by the results presented:

- Identifying qualitative and quantitative changes to amino acid composition in response to basal leaf removal in Sauvignon blanc grapes.
- How leaf removal modifies bunch microclimate, source/sink interactions and partitioning influences and effects amino acid accumulation in the grape.
- Potential mechanisms for the accumulation of the α-ketoglutarate amino acids; glutamine, glutamate, arginine and proline.
- The expression of genes involved in assimilation, biosynthesis, catabolism and regulation of the α-ketoglutarate amino acids.
- Activity of a grapevine OAT enzyme during berry development.

The results presented reflect a major study of amino acid biochemistry and gene expression specifically in grapevine and demonstrate a number of novel discoveries that are highlighted in the Results (Chapters 3-6) and Discussion (Chapter 7) sections. There is considerable opportunity to further investigate many of the novel observations highlighted by this research and they are discussed in the appropriate chapters and indicative of the potential for further research.

Such a research undertaking, obviously draws attention to results and findings not covered by the scope of the research presented. Such observations not addressed during this research are also mentioned in the appropriate sections as are their potential for further investigation. As previously discussed, the main focus of amino acid research presented here was the α-ketoglutarate family of amino acids. An obvious area of research that was not significantly addressed in this thesis for example, is the regulation of phenylalanine, as a precursor to phenolic secondary compounds and its relationship to berry biochemistry and subsequent wine quality. This is an interesting area of amino acid biochemistry and genetics that was only moderately examined in this study.

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Appendix

Appendix 1 Table of primers used for qPCR analysis.

	Primers (5'-3')	
Gene	**Forward**	**Reverse**
Flavonoid biosynthesis		
VvFLS4	CAGGGCTTGCAGGTTTTTAG	GGGTCTTCTCCTTGTTCACG
Reference genes		
VvGAPDH	TTCTCGTTGAGGGCTATTCCA	CCACAGACTTCATCGGTGACA
VvActin	CTTGCATCCCTCAGCACCTT	TCCTGTGGACAATGGATGGA
VvEF-1α	GAACTGGGTGCTTGATAGGC	AACCAAAATATCCGGAGTAAAAGA
VvSAND	CAACATCCTTTACCCATTGACAGA	GCATTTGATCCACTTGCAGATAAG

Appendix 2 Table of target probe sequences used for nCounter analysis.

Gene	Probe sequence
FLS4	5’_GTCGATCACCTCTTCCATAACATCTGGCCTCCTCCTGCCATTGACTATCAGTTCTG GCCTAAAAAACCACCTACTTACAGAGCTGCGAACGAGGAGTATG_3’
GS1-1	5’_GTGATTGTAGAGTACATATGGGTTGGTGGATCTGGTATGGACCTTAGAAGCAAA GCCAGGACCCTTTCTGGACCAGTTAGCGATCCTGCAAAGCTTCCCA_3’
GS1-2	5’_GCAACAATATTCTTGTCATGTGTGATACTTACACTCCTGCTGGGGAGCCCATTCC AACCAACAAGAGGCATAACGCAGCTAAAATATTTAGCCATCCTGA_3’
GS1-3	5’_TTTTATACCCTCAGGCGATCTTTAAGGATCCCTTCAGAGGAGGAGACAATATTCT GGTCATGTGCGATGCCTACACTCCAGGGGGCGAACCAATCCCGAC_3’
NADH-GOGAT-1	5’_CAAGTTGCAAGATGCTGCCAGATCTAATAGTGTGGCTGCCTACAAAGAATACTC CAAGCGCATACAGGAACTGAATAAAACCTGTAATTTGCGTGGACTT_3’
NADH-GOGAT-2	5’_AGCTCTATTGGAGCCATGGGATGGGCCTGCTCTTATATCATTTACCGATGGAGCT ATCTTTTCAAACAAGGTAATCAATGGCCCACAAGACAAAGGAAAC_3’
P5CS	5’_GGAAACACTTCTTGTACACAAGGATTTAGTGCAGACTGGTGGCCTCAATCAGCTT ATCGTAGAGCTCCGCAATGAAGGGGTTACTTTATATGGTGGACCA_3’
P5CS1a	5’_TTATTACCCATCGGGACCATCACCAACTCACTTTGGTGCCTATGTTCATCCTTATG CATTTACTCCACCACCACCATCTATTTATGGTGCTGCACCACCA_3’
P5CS1b	5’_AAAGAGAGGATCAAATTATAGAGAATCAGGAGGATCTTTGGTCTTGCCTTACCG TTATCTTTGGTGGAGTTCTTATGAAGGTGGTGAGAACTGTAACTGA_3’
P5CR	5’_AATGGCAGAGAGCATTGCGAAAGGAGTCGTCCATTCAGGCCTCATGCCCGCTTC CCGAATCTCTACTGCTCCTAGAAGTTCGAGTCGCCAAGATGCCTTT_3’
PDH	5’_ATCAGGGAAATTGCTAGCCACAAAAGCGCGAGACTTGGGAATTTCGAAGGAAG ACCACAAGGTCCAATTTGCTCAGCTATATGGAATGTCAGAGTCACTT_3’
P5CDH	5’_ATGCTGAAGGATGGCAATTATGAGCTTGTTACAAGAGAGATTTTTGGACCATTC CAGGTTGTAACTGACTATAAAGACAATCAGCTACCCAGGGTGTTGG_3’
Arginase	5’_ACGCCACAACTGAAGAAGGGAAAGAATTAAATGATCCACGGGTGCTTACTGATG TTGGTGATGTCCCTGTCCAAGAGATAAGAGATTGTGGTGTAGATGA_3’
OAT	5’_GACAGGAGCTTAGGAATCAGCTAATGAAGATTCATCAGCTATTCCCTAACCTCAT AAAGGAAGTGCGAGGAAAAGGTCTGTTCAATGCTGTGGAGCTCAA_3’
SNAC2	5’_CAAAATAAAGTAGTACCCTCTTCATTGCCGTCCCTACCTCCAATGCCTGCGCCGC CAACAACCAATGACTACCTATACTTCGAGACATCTGATTCAGTGC_3’
Actin	5’_GACCTTGCTGGACGTGACCTCACTGATGCTCTGATGAAGATTCTAACTGAGAGG GGTTACATGTTCACCACTACTGCTGAACGGGAAATTGTCCGGGACA_3’
GAPDH	5’_TTGGTGAGAAGCCAGTCACCGTCTTTGGTATTAGGAACCCAGAGGAGATTCCAT GGGGTGAGACCGGAGCTGAATTTGTTGTTGAGTCAACTGGTGTGTT_3’

Appendix 3 Amino acid concentrations in Sauvignon blanc grapes through berry development.

Amino acid (µmol/L)	Total amino acids - 2013 season				Glutamine - 2013			
Treatment	CANOPY	PRE	POST	*lsd*	CANOPY	PRE	POST	*lsd*
DPV								
-18	16430	15168	-	*3967*	11587	10902	-	*3950*
-8	14921	12766	-	*6627*	7949	6391	-	*3160*
-1	13619	*10047	-	*3167*	5515	4084	-	*2243*
6	17349	*10327	15830	*5988*	5349	3546	5216	*2479*
8	18112	11155	19846	*10220*	5229	3423	6648	*4771*
10	18458	11800	18015	*8535*	4888	3531	5274	*3568*
13	18178	10918	17817	*8750*	4239	2538	4828	*2946*
15	20916	* 9971	15972	*7033*	5101	*2114	3602	*2141*
17	23891	12600	17833	*11377*	5457	2516	4261	*3647*
20	21578	*11766	*14638	*4842*	4150	*1897	2883	*2043*
24	23365	*11725	*11853	*5129*	4385	*1944	*1869	*1663*
27	25009	*12326	*16862	*7073*	4658	*1648	*2668	*1582*
30	23933	*14480	17841	*9359*	3672	1812	2898	*2654*
37	22552	*13215	*13571	*8000*	2067	1043	1098	*1139*
44	21490	*15074	*15460	*5627*	1510	862	856	*799*
51	21977	18424	15469	*8224*	1258	1094	828	*756*
62	26110	20735	19756	*6789*	1183	1116	1177	*696*

Amino acid (µmol/L)	Glutamate - 2013				Arginine - 2013			
Treatment	CANOPY	PRE	POST	*lsd*	CANOPY	PRE	POST	*lsd*
DPV								
-18	1008	880	-	*241*	1035	930		*818*
-8	1013	946	-	*350*	2081	2075		*1557*
-1	946	*766	-	*152*	2568	*2010		*359*
6	999	*756	971	*191*	3386	*2074	2938	*1007*
8	1170	910	1195	*311*	3622	2466	3612	*1273*
10	1143	942	1194	*243*	3805	*2482	3556	*889*
13	1117	880	1173	*343*	3938	*2566	3904	*1336*
15	1179	*779	1004	*227*	4454	*2596	4080	*1366*
17	1254	922	1082	*372*	5332	3478	4403	*2144*
20	1287	*1021	*1040	*182*	4935	*3270	*4006	*364*
24	1106	* 809	* 855	*214*	5952	*3588	*3868	*1232*
27	1520	1146	1327	*384*	6416	*3900	5186	*1370*
30	1331	1121	1189	*339*	7137	*4782	5564	*2239*
37	1352	1152	1114	*436*	7650	*4577	5225	*2442*
44	1455	1375	1386	*301*	7790	*5607	6033	*2168*
51	1331	1432	1296	*377*	8407	6224	5885	*2598*
62	2054	1921	1895	*215*	9943	*6850	*6428	*2127*

Values are means (n = 3). *Indicates statistical differences using ANOVA and Fisher's least significant difference (*lsd*) test at the 5% level.

Amino acid (µmol/L)	Proline - 2013				Aspartate - 2013			
Treatment	CANOPY	PRE	POST	*lsd*	CANOPY	PRE	POST	*lsd*
DPV								
-18	58	23	-	*61*	473	408	-	*68*
-8	162	114	-	*125*	465	383	-	*193*
-1	149	96	-	*113*	547	*443	-	*99*
6	403	*167	298	*184*	518	459	534	*75*
8	443	207	461	*264*	352	363	417	*55*
10	545	302	491	*326*	518	*458	473	*58*
13	767	392	627	*525*	534	*478	570	*50*
15	929	*489	687	*389*	484	*394	456	*32*
17	1156	708	729	*545*	490	*445	483	*34*
20	1110	770	685	*522*	453	420	473	*65*
24	1240	*725	*649	*348*	445	415	385	*70*
27	1225	978	1086	*708*	384	304	350	*108*
30	1266	1236	1401	*746*	595	539	506	*127*
37	1682	1519	1244	*1035*	696	647	658	*160*
44	1913	1835	1816	*825*	408	346	353	*79*
51	1965	2792	1965	*1294*	540	591	553	*164*
62	2856	2901	2672	*659*	275	249	286	*99*

Amino acid (µmol/L)	Asparagine - 2013				Threonine -2013			
Treatment	CANOPY	PRE	POST	*lsd*	CANOPY	PRE	POST	*lsd*
DPV								
-18	443	387	-	*111*	222	185	-	*67*
-8	530	462	-	*254*	299	269	-	*138*
-1	427	*297	-	*110*	531	324	-	*233*
6	301	208	315	*148*	1183	* 575	972	*308*
8	311	236	340	*138*	1280	714	1263	*605*
10	295	177	299	*155*	1390	791	1245	*604*
13	233	131	253	*116*	1468	878	1340	*597*
15	283	*102	194	*132*	1660	* 882	1350	*574*
17	280	106	212	*175*	1937	*1114	1478	*639*
20	194	* 77	146	*96*	2030	*1122	*1353	*335*
24	214	* 79	* 94	*71*	2240	*1124	*1161	*488*
27	261	* 80	*160	*91*	2140	*1188	*1536	*466*
30	217	90	138	*129*	2346	*1356	1610	*821*
37	138	* 44	56	*83*	2297	*1248	*1292	*779*
44	100	* 40	47	*58*	2199	*1387	*1409	*567*
51	68	43	32	*56*	2221	1639	1373	*896*
62	89	73	78	*36*	2436	1732	*1585	*749*

Amino acid (µmol/L)	Isoleucine - 2013				Methionine - 2013			
Treatment	CANOPY	PRE	POST	*lsd*	CANOPY	PRE	POST	*lsd*
DPV								
-18	106	93	-	*39*	10	10	-	*6*
-8	80	64	-	*43*	30	24	-	*23*
-1	134	68	-	*120*	45	22	-	*46*
6	413	*104	316	*214*	136	* 26	109	*79*
8	453	123	431	*362*	159	33	172	*150*
10	511	176	423	*406*	190	55	151	*154*
13	562	*200	388	*340*	198	63	122	*140*
15	685	*147	*343	*331*	171	* 68	123	*68*
17	866	*252	450	*502*	280	*108	165	*152*
20	864	*286	*362	*279*	243	* 73	* 93	*82*
24	907	*239	*209	*301*	258	* 56	* 48	*91*
27	903	*195	*366	*428*	284	* 47	* 89	*189*
30	789	*258	*372	*357*	218	* 61	* 95	*120*
37	727	*174	*152	*299*	182	* 39	* 24	*87*
44	651	*262	*232	*174*	156	* 57	* 45	*54*
51	729	407	*269	*378*	161	94	* 50	*93*
62	856	576	475	*406*	167	101	* 71	*76*

Amino acid (µmol/L)	Lysine - 2013				Leucine - 2013			
Treatment	CANOPY	PRE	POST	*lsd*	CANOPY	PRE	POST	*lsd*
DPV								
-18	47	43	-	*28*	63	56	-	*30*
-8	169	165	-	*55*	181	154	-	*136*
-1	166	170	-	*18*	262	175	-	*118*
6	141	155	141	*17*	538	*183	412	*312*
8	141	150	143	*15*	595	187	572	*443*
10	138	150	140	*15*	675	240	567	*542*
13	128	132	124	*28*	740	*236	486	*482*
15	111	104	106	*15*	919	*189	*447	*421*
17	105	115	111	*13*	1177	*317	580	*689*
20	90	102	106	*20*	1120	*318	*433	*374*
24	119	98	89	*37*	1261	*299	*261	*472*
27	148	* 98	128	*24*	1252	*244	*465	*555*
30	170	*116	*134	*36*	1073	*328	*455	*483*
37	173	* 99	* 91	*58*	1039	*230	*211	*454*
44	165	129	*116	*41*	937	*341	*295	*270*
51	182	144	*116	*48*	1044	536	*346	*531*
62	234	196	162	*121*	1264	854	716	*616*

Amino acid (µmol/L)	Alanine - 2013				Valine - 2013			
Treatment	CANOPY	PRE	POST	*lsd*	CANOPY	PRE	POST	*lsd*
DPV								
-18	315	280	-	*132*	73	64	-	*9*
-8	515	434	-	*185*	90	64	-	*36*
-1	680	*401	-	*194*	133	58	-	*131*
6	1375	*598	1306	*427*	412	* 98	318	*222*
8	1597	814	1747	*856*	457	121	466	*394*
10	1455	800	1527	*783*	529	183	443	*421*
13	1383	786	1474	*824*	568	204	426	*378*
15	1694	*717	1342	*682*	700	*163	*366	*313*
17	1771	780	1363	*1040*	862	*270	475	*508*
20	1547	*708	* 976	*530*	809	*283	*354	*271*
24	1482	*742	* 797	*377*	832	*236	*208	*297*
27	1978	*965	1348	*800*	847	*216	*360	*463*
30	1619	985	1286	*785*	728	*271	384	*360*
37	1363	952	1006	*751*	678	*218	*189	*279*
44	1252	1035	1123	*645*	614	*307	*281	*169*
51	912	1060	890	*738*	700	471	323	*379*
62	1087	1310	1573	*779*	841	642	558	*377*

Amino acid (µmol/L)	Phenylalanine - 2013				Tryptophan - 2013			
Treatment	CANOPY	PRE	POST	*lsd*	CANOPY	PRE	POST	*lsd*
DPV								
-18	97	86	-	*4*	99	85	-	*28*
-8	136	104	-	*54*	152	142	-	*73*
-1	200	102	-	*132*	195	*144	-	*40*
6	473	*153	389	*222*	294	*168	260	*93*
8	525	176	521	*391*	314	187	315	*161*
10	582	225	497	*399*	330	205	305	*145*
13	620	*247	451	*347*	332	*199	305	*132*
15	732	*201	*397	*301*	369	*156	261	*151*
17	917	*306	490	*466*	429	*203	298	*195*
20	924	*348	*428	*255*	395	*207	*256	*94*
24	1019	*332	*295	*310*	408	*180	*195	*106*
27	1010	*296	*446	*353*	414	*161	*242	*164*
30	919	*364	*447	*385*	373	*185	231	*146*
37	837	*298	*247	*318*	320	*142	*141	*106*
44	778	*412	*354	*186*	286	*170	*170	*73*
51	887	560	*412	*377*	324	*191	*162	*128*
62	1050	763	687	*389*	336	223	226	*135*

Amino acid (µmol/L)	Tyrosine - 2013				Histidine - 2013			
Treatment	CANOPY	PRE	POST	*lsd*	CANOPY	PRE	POST	*lsd*
DPV								
-18	43	40	-	*26*	158	134	-	*37*
-8	118	109	-	*87*	268	240	-	*146*
-1	150	125	-	*36*	279	235	-	*46*
6	183	139	174	*160*	319	235	283	*95*
8	194	139	204	*67*	317	*225	302	*87*
10	192	144	195	*68*	293	222	272	*74*
13	180	*120	177	*38*	276	194	266	*94*
15	125	76	135	*142*	295	*175	225	*83*
17	186	90	150	*109*	302	*195	232	*96*
20	167	* 76	114	*61*	265	*185	*212	*24*
24	171	* 71	* 85	*45*	297	*180	*182	*49*
27	172	* 63	112	*77*	302	*168	213	*109*
30	154	82	100	*89*	282	194	228	*104*
37	132	* 52	* 53	*57*	251	*160	*162	*77*
44	121	87	91	*34*	242	*167	*173	*57*
51	143	118	100	*65*	228	192	168	*65*
62	183	148	142	*63*	250	205	198	*60*

Amino acid (µmol/L)	Serine - 2013				Glycine - 2013			
Treatment	CANOPY	PRE	POST	*lsd*	CANOPY	PRE	POST	*lsd*
DPV								
-18	503	497	-	*169*	65	65	-	*34*
-8	567	*477	-	*70*	115	147	-	*74*
-1	559	*355	-	*202*	134	172	-	*89*
6	772	*458	721	*185*	155	*224	156	*58*
8	814	529	901	*341*	137	154	135	*43*
10	867	563	847	*318*	112	153	117	*48*
13	804	552	802	*315*	90	*121	102	*16*
15	903	*512	756	*287*	123	107	100	*48*
17	990	580	787	*440*	100	97	84	*32*
20	902	*513	*639	*218*	92	89	80	*10*
24	939	*535	*541	*252*	89	75	63	*45*
27	1005	*564	717	*352*	91	65	63	*33*
30	948	627	730	*376*	94	71	72	*39*
37	872	555	549	*348*	95	67	58	*37*
44	819	588	618	*234*	95	64	64	*41*
51	789	765	645	*382*	86	72	55	*36*
62	914	797	741	*249*	92	78	87	*40*

Amino acid (µmol/L)	Total amino acids - 2014 season				Glutamine - 2014			
Treatment	CANOPY	PRE	POST	*lsd*	CANOPY	PRE	POST	*lsd*
DPV								
-23	13156	15110	-	*3694*	10035	11454	-	2717
-16	14525	16003	-	*5791*	10039	10345	-	2246
-9	13425	13334	-	*3138*	7773	7616	-	1232
-2	14185	12511	-	*3574*	6477	*5625	-	637
5	15973	12384	-	*4433*	5488	*4438	-	357
8	17367	*12929	-	*3083*	5618	4337	-	2383
12	14852	*12026	14876	*2823*	4470	3804	4506	1265
16	16615	*11707	17496	*1804*	4335	3097	4561	1364
19	18762	*12224	17280	*3369*	4572	3242	3845	2386
23	17170	*11492	16386	*3260*	3038	2215	3081	1787
26	17037	*12624	14536	*4112*	2913	2426	2287	734
29	16951	*13158	15652	*1765*	2649	2496	2459	1327
33	18419	*11829	*16025	*2384*	2636	1768	2247	873
40	17765	*12761	*13680	*3930*	1711	1451	1188	1429

Amino acid (µmol/L)	Glutamate - 2014				Arginine - 2014			
Treatment	CANOPY	PRE	POST	*lsd*	CANOPY	PRE	POST	*lsd*
DPV								
-23	751	872	-	269	358	441	-	175
-16	857	1002	-	556	1001	1475	-	1289
-9	808	829	-	274	1685	1804	-	1102
-2	880	819	-	241	2305	2223	-	1321
5	1249	*1038	-	165	3019	2571	-	1370
8	1257	1003	-	292	3594	2935	-	1897
12	1085	* 826	966	201	3399	2948	3499	1126
16	1224	* 914	1207	134	4212	*3276	4638	559
19	1477	* 925	*1244	139	4816	*3416	5056	267
23	1661	*1262	*1370	247	4801	*3402	5337	1112
26	1216	* 844	1004	272	5341	4288	5243	1380
29	1262	* 926	*1089	59	5742	*4609	6002	771
33	1395	*1022	1209	314	6310	*4219	6237	1064
40	1366	986	1008	384	6578	4901	5913	2147

Values are means (n = 3). *Indicates statistical differences using ANOVA and Fisher's least significant difference (*lsd*) test at the 5% level.

Amino acid (µmol/L)	Proline - 2014				Aspartate - 2014			
Treatment	CANOPY	PRE	POST	*lsd*	CANOPY	PRE	POST	*lsd*
DPV								
-23	65	69	-	*16*	358	419	-	*120*
-16	54	73	-	*93*	389	476	-	*275*
-9	56	58	-	*26*	441	444	-	*212*
-2	91	100	-	*153*	555	486	-	*207*
5	285	200	-	*486*	545	469	-	*127*
8	434	299	-	*595*	607	564	-	*97*
12	317	317	385	*281*	587	533	550	*77*
16	606	411	594	*379*	640	*562	618	*56*
19	711	*414	722	*208*	696	*567	617	*85*
23	876	*462	642	*379*	656	608	593	*69*
26	985	720	820	*389*	729	*622	*615	*68*
29	1061	741	874	*463*	551	*459	515	*48*
33	1306	772	861	*671*	535	497	528	*69*
40	1505	974	965	*693*	620	554	545	*109*

Amino acid (µmol/L)	Asparagine - 2014				Threonine - 2014			
Treatment	CANOPY	PRE	POST	*lsd*	CANOPY	PRE	POST	*lsd*
DPV								
-23	318	379	-	*51*	129	171	-	*91*
-16	394	470	-	*166*	187	246	-	*193*
-9	377	383	-	*24*	244	248	-	*120*
-2	353	*300	-	*47*	460	416	-	*550*
5	296	211	-	*85*	887	694	-	*666*
8	286	203	-	*107*	1042	816	-	*699*
12	227	175	228	*57*	948	786	1006	*334*
16	213	*145	234	*41*	1156	* 853	1308	*164*
19	233	147	211	*98*	1366	* 880	1349	*104*
23	180	113	176	*77*	1319	* 824	1238	*253*
26	151	*103	124	*46*	1365	* 983	1184	*284*
29	139	105	135	*58*	1404	*1072	1241	*201*
33	131	* 84	119	*37*	1517	* 968	1260	*277*
40	89	66	66	*57*	1431	1062	1101	*447*

Amino acid (µmol/L)	Isoleucine - 2014				Methionine - 2014			
Treatment	CANOPY	PRE	POST	*lsd*	CANOPY	PRE	POST	*lsd*
DPV								
-23	58	72	-	*28*	5	7	-	*5*
-16	98	123	-	*89*	20	24	-	*28*
-9	136	140	-	*67*	40	38	-	*26*
-2	179	163	-	*79*	60	*44	-	*7*
5	213	140	-	*113*	72	25	-	*54*
8	245	*150	-	*84*	80	*25	-	*30*
12	214	145	211	*74*	63	*26	58	*27*
16	272	*141	256	*107*	80	*20	81	*40*
19	344	*160	259	*135*	107	*26	77	*51*
23	312	*136	232	*108*	98	*20	*62	*27*
26	281	*143	*186	*69*	97	*27	*49	*34*
29	272	*149	*175	*49*	87	*27	*46	*15*
33	303	*136	*195	*28*	101	*18	*47	*18*
40	312	177	*164	*139*	97	*36	*35	*57*

Amino acid (µmol/L)	Lysine - 2014				Leucine - 2014			
Treatment	CANOPY	PRE	POST	*lsd*	CANOPY	PRE	POST	*lsd*
DPV								
-23	14	17	-	*4*	36	45	-	*17*
-16	22	28	-	*21*	90	116	-	*124*
-9	26	31	-	*16*	165	170	-	*118*
-2	29	33	-	*4*	238	209	-	*70*
5	21	24	-	*7*	244	153	-	*125*
8	29	37	-	*7*	284	*149	-	*91*
12	23	32	24	*3*	252	*154	243	*97*
16	23	21	22	*3*	335	*141	293	*135*
19	23	25	27	*6*	418	*164	294	*198*
23	26	39	36	*9*	371	*138	269	*124*
26	34	38	33	*4*	341	*146	*223	*101*
29	33	37	37	*3*	330	*149	*213	*36*
33	36	35	46	*1*	373	*130	*240	*48*
40	38	36	39	*8*	393	*192	*184	*178*

Amino acid (µmol/L)	Alanine - 2014				Valine - 2014			
Treatment	CANOPY	PRE	POST	*lsd*	CANOPY	PRE	POST	*lsd*
DPV								
-23	200	215	-	*27*	51	59	-	*16*
-16	235	332	-	*244*	81	99	-	*80*
-9	364	357	-	*200*	98	86	-	*64*
-2	888	615	-	*971*	173	123	-	*123*
5	1568	841	-	*1018*	266	132	-	*157*
8	1649	829	-	*914*	296	*138	-	*157*
12	1261	*748	1238	*423*	248	*140	250	*92*
16	1295	*696	1422	*223*	313	*138	312	*104*
19	1520	*788	1354	*325*	382	*160	308	*128*
23	1514	*912	1326	*321*	345	*150	276	*83*
26	1403	*867	1108	*475*	319	*155	*222	*90*
29	1323	*960	1204	*124*	311	*162	*213	*26*
33	1423	*928	1284	*253*	352	*149	*244	*56*
40	1457	*990	1120	*428*	369	202	202	*175*

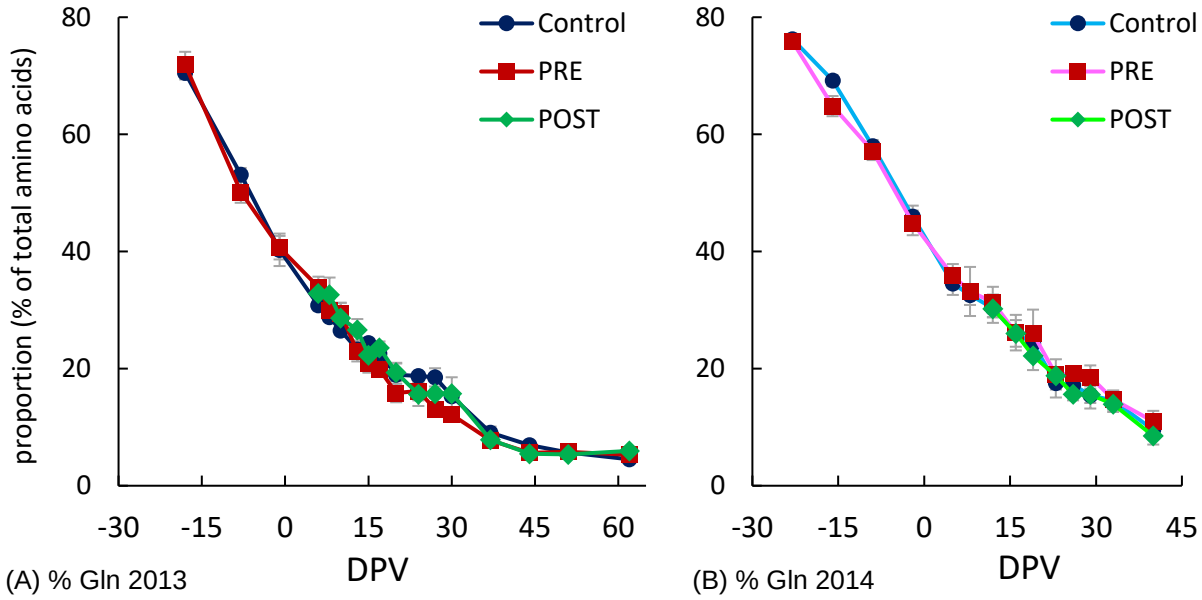
Amino acid (µmol/L)	Phenylalanine - 2014				Tryptophan - 2014			
Treatment	CANOPY	PRE	POST	*lsd*	CANOPY	PRE	POST	*lsd*
DPV								
-23	68	79	-	*17*	70	83	-	*36*
-16	86	84	-	*53*	91	109	-	*63*
-9	113	100	-	*41*	103	111	-	*67*
-2	188	137	-	*53*	142	122	-	*35*
5	288	146	-	*166*	172	122	-	*54*
8	336	*151	-	*86*	198	*126	-	*26*
12	305	*164	291	*93*	180	*118	180	*37*
16	372	*165	361	*105*	199	*101	203	*36*
19	446	*197	367	*137*	219	*108	195	*73*
23	436	*172	*305	*101*	203	* 96	168	*38*
26	404	*191	*261	*110*	186	* 95	*141	*43*
29	409	*184	*252	*32*	180	* 94	*142	*23*
33	459	*163	*275	*51*	196	* 77	*134	*16*
40	454	*230	*241	*196*	179	* 88	*101	*60*

Amino acid (µmol/L)	Tyrosine - 2014				Histidine - 2014			
Treatment	CANOPY	PRE	POST	*lsd*	CANOPY	PRE	POST	*lsd*
DPV								
-23	26	33	-	*14*	132	154	-	*46*
-16	64	86	-	*64*	162	203	-	*155*
-9	98	109	-	*45*	183	193	-	*114*
-2	153	164	-	*45*	203	213	-	*57*
5	164	159	-	*36*	195	195	-	*51*
8	183	156	-	*34*	194	*173	-	*15*
12	162	149	166	*34*	167	162	166	*28*
16	173	*127	178	*14*	195	*155	195	*32*
19	182	*125	165	*48*	213	163	180	*59*
23	153	* 99	146	*32*	177	223	262	*65*
26	141	*102	124	*39*	282	227	229	*61*
29	138	* 99	126	*19*	274	*236	258	*23*
33	145	* 86	*123	*16*	287	*203	*214	*34*
40	138	89	99	*59*	227	176	153	*83*

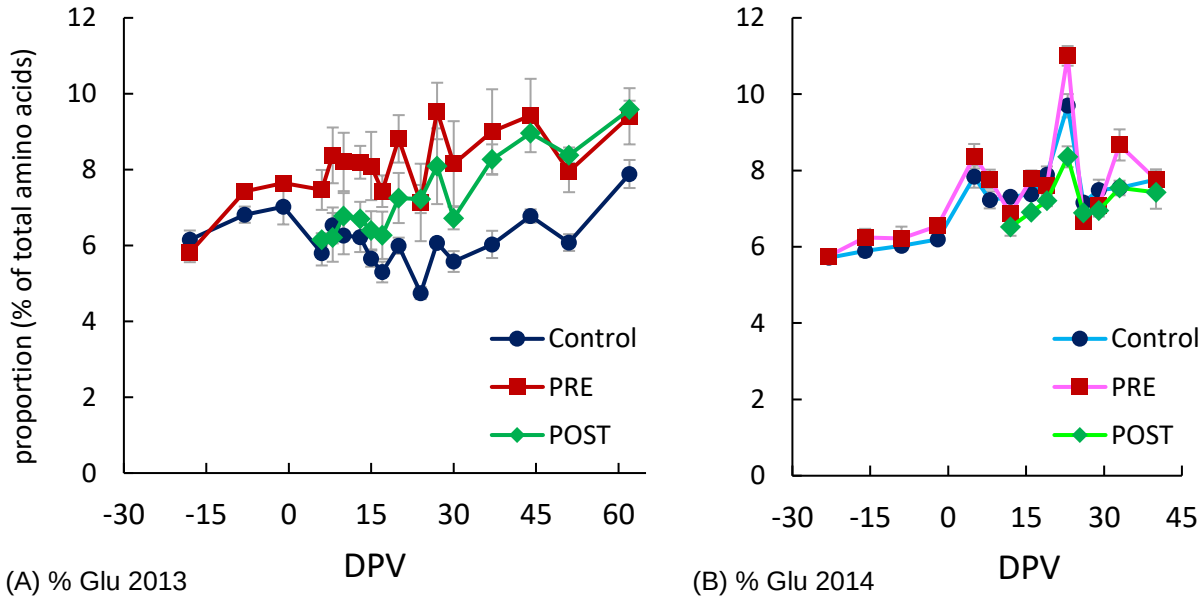
Amino acid (µmol/L)	Serine - 2014				Glycine - 2014			
Treatment	CANOPY	PRE	POST	*lsd*	CANOPY	PRE	POST	*lsd*
DPV								
-23	412	469	-	*124*	54	65	-	*48*
-16	554	543	-	*160*	95	138	-	*51*
-9	580	464	-	*188*	124	152	-	*41*
-2	654	516	-	*251*	125	*204	-	*71*
5	841	620	-	*270*	117	*205	-	*42*
8	885	*627	-	*141*	112	*191	-	*77*
12	806	*612	782	*180*	97	*161	105	*52*
16	855	*604	877	*89*	81	*118	83	*33*
19	915	*595	891	*65*	70	109	75	*45*
23	895	*552	757	*146*	58	67	59	*28*
26	773	*550	626	*164*	53	* 81	49	*7*
29	718	*561	*628	*44*	52	69	43	*17*
33	789	*508	672	*139*	56	65	53	*15*
40	735	498	517	*294*	43	43	39	*23*

Appendix 4 Qualitative effects of basal leaf removal on the α-ketoglutarate (glutamine, glutamate, arginine and proline) amino acids.

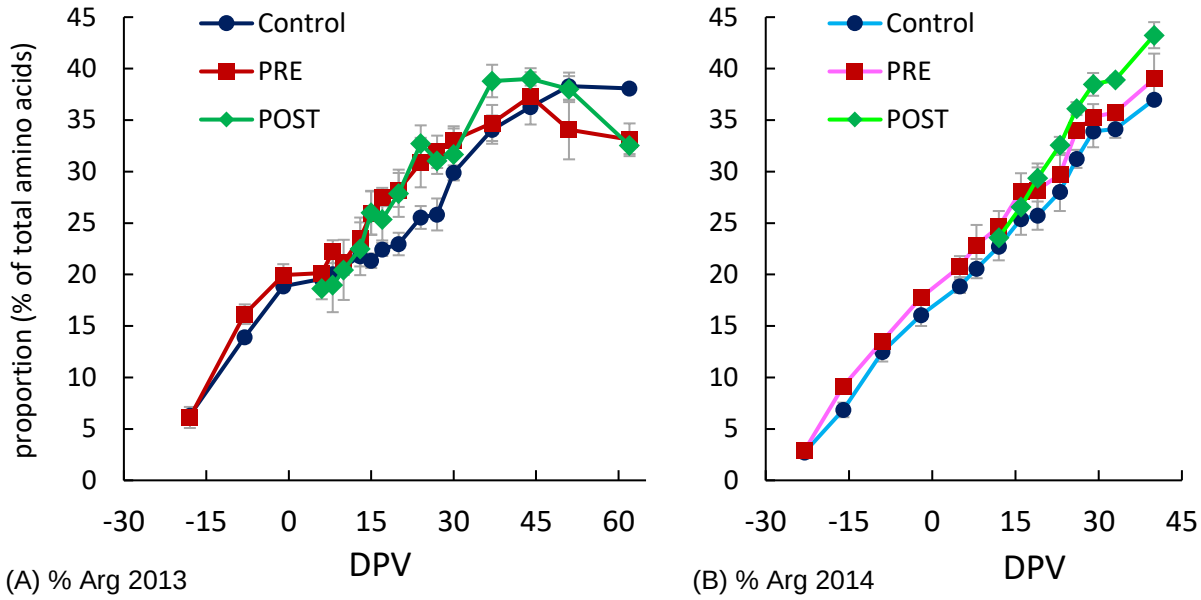
Proportions of the α-ketoglutarate (α-kg) amino acids in Sauvignon blanc grapes during berry development during the (A) 2013 season and (B) 2014 season. Relative proportions were calculated as percentages of total amino acid concentrations, with respect appropriate treatment, CANOPY control and (PRE and POST) leaf removal treatments. Sampling times are represented with respect to veraison (DPV, days postveraison). Each data point is the mean ± SEM (n = 3).



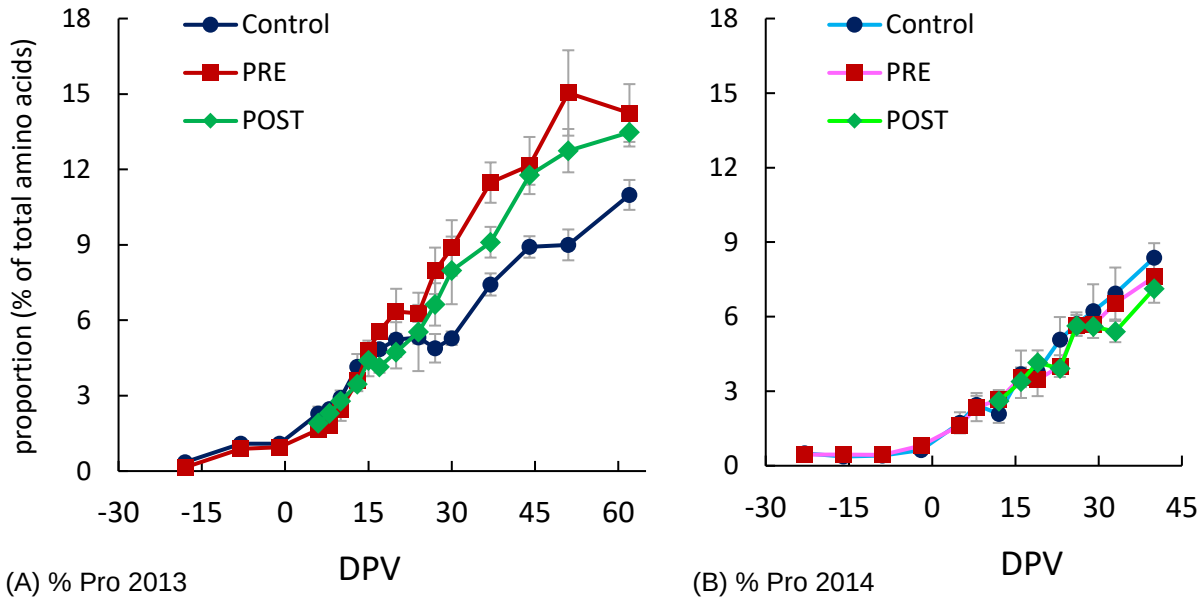
Appendix 4a Qualitative effects of basal leaf removal on glutamine accumulation.



Appendix 4b Qualitative effects of basal leaf removal on glutamate accumulation.



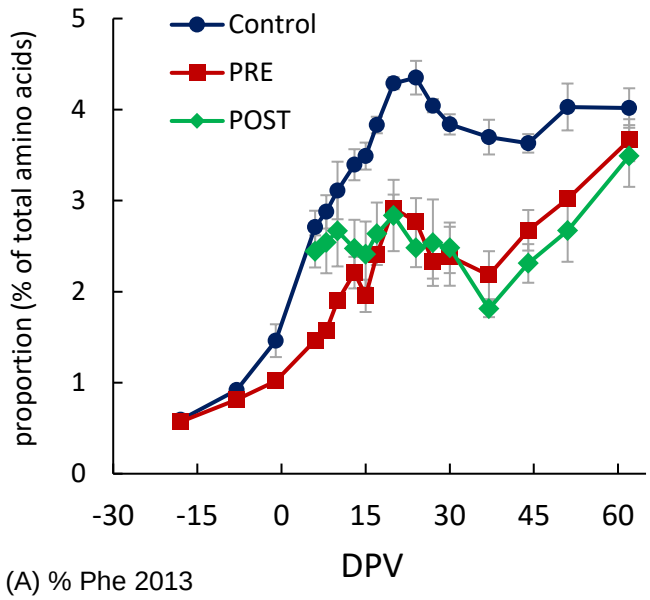
Appendix 4c Qualitative effects of basal leaf removal on arginine accumulation.



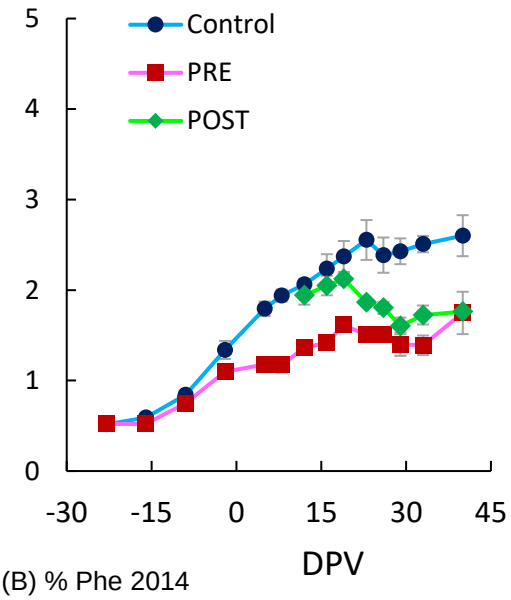
Appendix 4b Qualitative effects of basal leaf removal on proline accumulation.

Appendix 5 Qualitative effects of basal leaf removal on phenylalanine.

Proportions of phenylalanine in Sauvignon blanc grapes during berry development during the (A) 2013 season and (B) 2014 season. Relative proportions were calculated as percentages of total amino acid concentrations, with respect appropriate treatment, CANOPY control and (PRE and POST) leaf removal treatments. Sampling times are represented with respect to veraison (DPV, days postveraison). Each data point is the mean ± SEM (n = 3).



(A) % Phe 2013



(B) % Phe 2014